MICROGLIA DIFFERENTIATION AND CELL THERAPY

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1. Introduction

Aims

Microglia Therapy: Diseases like Alzheimer or Multiple Sclerosis have been linked to a continuous deterioration of the resident microglia cell population [1]. The brain, like other organs, also suffers from age related inflammation (“inflamm-aging”) [2] and chronic activation of microglia.

A cell therapy could aim to replace deteriorated microglia or to regulate the excessive activation of microglia in age.

We wanted to differentiate microglia from adult stem cells (**Paper 1 and 2**) and to proof their function (phagocytosis, oxidative burst, migration). Then, we planned to transplant their progenitors and the differentiated microglia (**Paper 3 and additional data**). We also wanted to transplant mesenchymal stem cells (MSC) since MSC are known to regulate inflammation [3].

We aimed to:

1. Differentiate microglia from mouse bone marrow and proof their function (**Paper 1 and 2**)
2. Observe migration, survival, morphology in co-culture with brain tissue (**Paper 1 and 2**)
3. Transplant microglia progenitors, microglia and MSC, track cells and stereological measurement of microglia numbers and activity (**Paper 3 and additional data**)

Differentiation of Microglia: For transplantation of microglia progenitors and mature microglia, it is necessary to prove the capacity to differentiate to functional microglia (**Paper 1 and 2**). For application in a cell therapy we have not only to prove phenotype (Morphology, markers) but also the function of these cells (Phagocytosis, oxidative burst).

Several protocols exist in literature to differentiate microglia [4-7].

They have been differentiated from peripheral blood monocytes [4], bone marrow [5, 6] and from embryonic stem cells (ESC) [7] (Table 1). All of these protocols make use of the non-adherent fraction of bone marrow cells. Primary microglia can be isolated from the brain and the central nervous system (CNS).

Sucessful differentiation has been tested in literature by display of the typical microglia morphology (Ramification) and the expression of non-exclusive markers [6]. Furthermore, as a proof of principle, we planned to test the capacity of the differentiated cells to perform functions of primary microglia (Phagocytosis, oxidative burst) before use in transplantation.

Author and year	**Cell source**	**Differentiation protocol**
Leone 2006	Human peripheral blood monocytes (PBMC)	ACM, GM-CSF, M-CSF
Davoust 2006	Mouse CD34+/B220+ bone marrow cells (BMC)	M-CSF, ACM
Servet-Delprat 2002	Flt3L treated non-adherent BMC	Flt3L, ACM
Tsuchiya 2005	Mouse ESC	ITSFn, bFGF, laminin, GM-CSF

Table 1: Overview of differentiation methods for microglia.

Peripheral blood monocytes have been differentiated to microglia by Leone et al. [4] using astrocyte conditioned medium (ACM), granulocyte-monocyte colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF). They observed that addition of LPS returned morphology and levels of substance P to that of monocytes. It had been known that co-culture with astrocytes differentiates blood monocytes to microglia [8]. Furthermore, it has been repeatedly noted that GM-CSF expands primary microglia stronger than M-CSF, for example by Lee et al. [9]. In lesions of Multiple Sclerosis and in Alzheimer plaques astrocytes are known to produce GM-CSF and regulate microglia proliferation. Primary microglia can be differentiated with M-CSF to display a rod shaped morphology [9]. Tsuchiya et al. used a modified 5 step protocol for neuronal differentiation to derive microglia from ESC [7].

Here, we focused on differentiating microglia from bone marrow. This approach was first demonstrated by Servet-Delprat et al. [6]. The group obtained 20% of the cells showing microglia-like morphology and displaying microglia markers (CD115+, CD11b+, F4/80+, CD80 low, CD86-). In the paper, bone marrow cells from the mouse were cultured with FMS-like tyrosine-kinase-3 ligand (Flt3L) for 11 days. Afterwards, the supernatant was treated with astrocyte-conditioned medium for 6 days. The effect of Flt3L on the differentiation of microglia remained unclear because supplementation of Flt3L was not controlled.

A similar protocol was used by Davoust et al. [5]. However much shorter culture times and no Flt3L were used to obtain $CD11b^+$, $CD45^+$, $MHCII^-$, $B220^{low}$, $CD34^+$, $CD86^{low}$ cells from mouse bone marrow. Roughly 20% ramified and 50% nestin positive cells could be obtained.

It was intriguing to investigate the impact of Flt3L, as done by Servet-Delprat et al. [6], for differentiation of microglia. We therefore wanted to test the combination of ACM and Flt3L in a modified protocol and on whole bone marrow. The effect of ACM on bone marrow both alone and in combination with the cytokines stem cell factor (SCF) [10] and GM-CSF has been reported to be advantageous for microglia differentiation.

Non-adherent bone marrow cells (NA-BMC), defined according to Zhang et al. [11], might correspond to a circulating cell population and contain progenitors of many different cell types. The capacity of NA-BMC to differentiate to microglia has not been investigated until now and we wanted to test their potential for such differentiation.

Furthermore, the flat influence of *in vitro* culture time, as well as cell density, on the appearance of phagocytic cells plays an important role in differentiation. We also modified a macrophage differentiation protocol [12] and supplemented ACM instead of M-CSF.

Co-culture with Living Brain Tissue: We planned to prove in pre-transplantation studies that microglia derived from adult stem cells migrate into living brain slices *in vitro*, survive in co-culture and integrate (ramify) (**Paper 1 and 2**).

Differentiated microglia, if they are to be used in transplantation, have to be able to invade and integrate into brain tissue. It is not granted that the differentiated cells survive for a prolonged time in co-culture [13]. There might be rejection by the brain's own microglia [14]. Also, in brain slices it can be studied whether the seeded cells are able to perform functions, for example phagocytosis of dead cells [15]. After a while, the seeded cells might cease their activity and integrate into the brain tissue, possibly even becoming ramified, similar to the resting state of the brains own microglia [16].

Living brain slice cultures (Organotypic hippocampal slice cultures (OHSC)) were first established by Stoppini et al. [17]. They were cut from newborn (1-20 days old) mice brains and it became quickly apparent that young brains were better suited to cope with the injury of cutting the brains into slices. The brain slices reverted to an organotypic environment about 9 days after being cut. These brain slices contain functional neurons that show electric currents in patch clamp experiments [18]. Cultured brain slices from older mice have been reported not to display such currents. However, there are also claims that the tissue of brain slices from older animals is nontheless alive [19]. We wanted to use living brain slices for migration and co-culture experiments accompanying our transplantation experiments. We aimed to establish living brain slices of older animals (2-3 months) to have *in vitro* tissue for migration and co-culture comparable to the *in vivo* transplantation situation.

Transplantation and Cell Tracking: We planned to transplant and track microglia progenitors and MSC **(Paper 3 and Additional Data)**.

Progenitor cells from the bone marrow might migrate through the blood stream to the brain and mature there to microglia [20]. Pronounced migration has been shown in certain cases of disease, injury and irradiation of the brain. We therefore planned to transplant microglia progenitors systemically in aged mice. As progenitor population for transplantation, we chose NA-BMC defined according to Zhang et al. [11]. NA-BMC contain immature cells, both MSCs but also hematopoietic stem cells (HSC) [11].

MSCs derived from bone marrow should be transplanted to investigate a possible regulation of microglia *in vivo*. With advancing age inflammation, and possibly also microglia activity, rise [2].

MSCs are known to establish locally a suppressed immune environment and, in large numbers, also suppress the immune system organism wide [3, 21]. For example it is known that large amounts of fat tissue containing MSCs suppress the immune system (WCRF study: Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective) [22]. The immune suppression effects by MSCs could be useful to treat chronic inflammation and autoimmune diseases [23].

Stereological Measurements: We planned to quantify changes in microglia numbers and activation in response to cell transplantation **(Additional Data)**. We also wanted to quantify changes between young and aged animals. Microglia numbers have been reported to rise in female mice in age. This rise might be caused by a difference in sex hormones or by general inflammation [24]. Also microglia activity was observed to rise in age. This rise in inflammation in various aged tissues might be a general phenomenon, often called ‘inflamm-aging’ [2]. Connected to this, changes in the microenvironment of several tissues in age are documented.

Fundamentals

Microglia: Microglia have first been described by Del Rio Hortega in 1919 [25]. They comprise about 10% of the cell population of the brain [26]. Microglia form the first immune defense of the CNS. They perform phagocytosis, act cytotoxic and they present antigens. Microglia promote repair after injury [20].

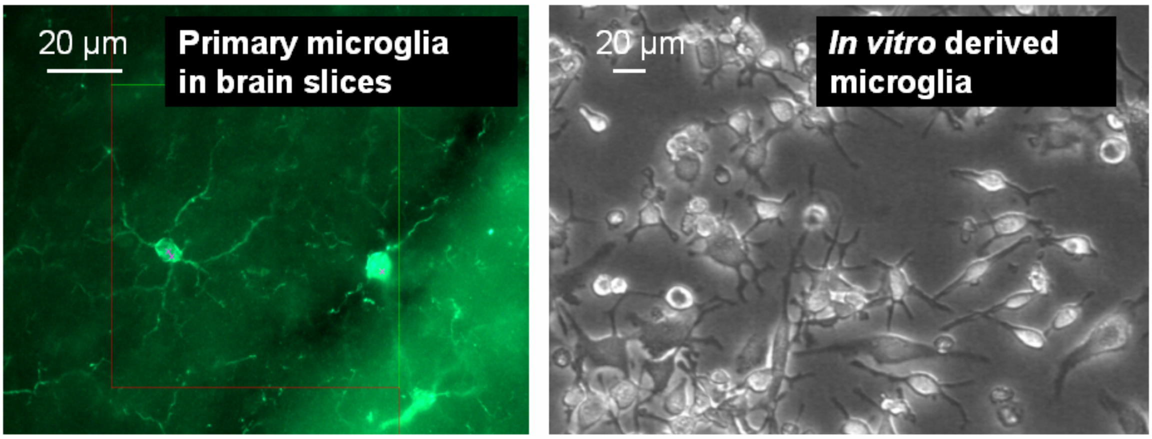

Fig. 1: Microglia *in vivo* and *in vitro*. Microglia on the left are stained with Iba-1 in 40 µm thick brain slices. Pictures were taken with an Imager A1 (Leica) fluorescence microscope at 630x. The light microscope picture on the right was taken with a DM IL (Leica) light microscope (200x).

In general, microglia display a quiescent state in the brain (Fig. 1). Phagocytosis, immune response and migration are down-regulated in this state. The microglia are ramified and display long and branched processes [27, 28].

Inflammation causes microglia activation and a change to an amoeboid morphology. Microglia also start to proliferate in reaction to inflammation [29-31]. They migrate towards injury, lesions and extracellular debris, for example amyloid-β plaques [32].

The “oxidative burst” is one of the hallmark microglia functions. Microglia perform a sudden spike in reactive oxygen species (ROS) levels caused by NADPH oxidase activity in response to inflammatory signals. Also, other factors like lysosomal proteases are released. This mechanism is a typical feature of microglia and often nicknamed a 'defense' response to protect the brain from pathogens [33, 34].

Also, microglia play an important role in development and restructuring of neural networks. Microglia purge axonal connections [35]. Furthermore, they carry specific receptors to detect tagged synapses and remove them [36].

During development, microglia also act pro-apoptotic. They remove apoptotic neurons and excess connections [37].

Origin of Microglia: Microglia might arise from a variety of sources and, despite ongoing research, their exact origin is still unknown [38]. Microglia might be of neuroectodermal origin, might arise from the vascular adventitia, from HSC intrinsic to the central nervous system (CNS), from mesodermal tissue or from circulating monocytes. There are reports that primary microglia might home to the brain while macrophages do not [39-41]. Therefore it might be of great therapeutical interest to identify the originating tissue [38]. However, there have been controversial findings regarding directed microglia homing to the brain.

It has gained a certain acceptance that microglia in the CNS originate from a mesodermal progenitor cell during embryogenesis [38]. The CNS is populated by these progenitors during development. This takes place on (embryonic) days 10-19 in rodents [42]. In man, this colonization takes place between pre-natal months 3-5. This early population might arise both from myeloid/mesenchymal origin and from fetal macrophages [42]. During later life, additional myeloid progenitors might migrate from the bone marrow and invade the brain [20]. In the brain such microglia progenitors mature under the influence of factors secreted by astrocytes [8]. In this way a slow turnover and replenishment of the microglia population might take place. In line with this hypothesis, bone marrow derived microglia can be observed in the brain after systemic transplantation [43]. However, while bone marrow (BM) chimeras have shown BM derived microglia [44], other findings indicate that without irradiation no invasion is observable over a time frame of 1-2 months [45, 46]. Other groups could observe migration of intravenously injected hematopoietic stem cells to the brain also in non-irradiated mice [47]. They differentiated into microglia and reduce infarct size [47].

Microglia are capable to proliferate locally and do not necessarily require turnover [45, 48]. Others have challenged this view and hypothesized that microglia reach replicative senescence in later life [1]. Furthermore, a loss of function of aged microglia has been shown [1, 49-54].

Microglia in Disease: The general quiescence and graded up-regulation of microglia activity might exist to minimize damage to neighboring neural tissue if an immune response is initiated [1]. However, if microglia are chronically activated, they act cytotoxic and damage cells in the vicinity [55]. Furthermore, microglia might play a central role in autoimmune diseases of the CNS.

Whether microglia act harmful or beneficial in several diseases of the CNS is controversial [1, 56, 57]. Both ways of action have been shown in organotypic hippocampal slice cultures (OHSC). When microglia were co-cultured with brain tissue and treated with lipopolysaccharides (LPS) they acted neurotoxic. Contrarily, if they were activated with interleukin 4 (IL-4), the microglia acted neuroprotective in co-culture. IL-4 activated microglia up-regulate IGF-1 and down regulate tumor necrosis factor alpha (TNF-alpha) [49, 58].

The role of microglia seems to depend on distinct sets of surface receptors involved in phagocytosis. Receptors recognizing microbes, for example toll like receptors (TLR), stimulate a pro-inflammatory response. Microglia activated in this way excrete signals associated with inflammation, like tumor necrosis factor (TNF), interleukin IL-1 and nitric oxide (NO).

However, if microglia encounter cell debris, receptors recognizing phosphatidylserine in apoptotic membranes drive their response. Microglia then perform a ‘silent’ phagocytosis [49] and act anti-inflammatory. They show anti-inflammatory cytokines like tumor growth factor beta (TGF-beta) and interleukin 10 (IL-10) [59].

Also, the loss of function of certain receptors like the triggering receptor (TREM2) or DNAX-activating protein 12kDa (DAP12) has been observed to lead to an inflammatory neurodegenerative disease later in life [60].

Under pathological conditions, for example infection, stroke or neurodegeneration, microglia are activated either by direct contact with debris or microbes or by cytokines. They migrate to the site of injury. There, the microglia release soluble factors, for example cytotoxins, neurotrophins and immunomodulatory molecules. They also perform phagocytosis. It becomes apparent that microglia act both destructive and also promote repair and regrowth.

Normally, microglia are ramified. It had long been thought that in this state microglia are virtually non-motile. However, *in vivo* motion picture observations have shown that the processes are in fact continuously forming protrusions and are extending and withdrawing [27]. This is reminiscent of extensions in macrophages, which act to draw debris to the cell body for phagocytosis. Similar behavior can be observed in microglia. It is suspected that the long and branched processes in ramified microglia monitor the local neighborhood in this way. In line with the different routes of activation through distinct receptors mentioned above, the removal of 'normal' debris takes place without full activation or strong movement of the cell body. However, if microglia are completely activated, they withdraw processes and the whole cell body moves to injury or plaques [32].

If an injury is caused, for example by facial nerve axotomy, initially local neuroinflammation and enhanced proliferation of microglia take place. The regeneration of axons is completed over a time frame of about 4 weeks. At this time, the microglia activity has already ceased [1].

Microglia in Age: The microglia cell population proliferates locally, but might additionally be replenished by bone marrow derived progenitors, which migrate over the circulation to the brain [1].

Resident microglia might reach replicative senescence in old age [1]. In old rats there have been indications that proliferation of microglia after injury is stronger than in young rats [61]. *In vitro*, microglia have been reported to undergo telomere shortening [62]. *In vivo,* however, there are reports of stable telomeres and higher telomerase activity of microglia after their proliferation had been induced by injury [63].

This has been linked to the development of neurodegenerative diseases, especially Alzheimer, in humans but not in relatively short lived rodents. Over the course of such short live spans, a replicative senescence and complementary deterioration of the microglia cell population might simply not take place [1]. In brains of older humans, deterioration of microglia is observable. In the aging human brain, dystrophic microglia are present which show deramification, atrophy, fragmentation and swelling [62]. *In vitro* cultured microglia, if exposed to amyloid protein, are degenerating faster [64]. This has also been reported by others, and an overload of the intracellular mechanisms to break down digested proteins (proteasome) might be one of the possible causes [49, 50, 54].

However, even without additional stress by plaque overload or continuous activation, there is evidence that a slow deterioration of the microglia cell population in age takes place. Aged microglia have been observed to loose their ability to perform normal microglia functions [1, 49-54].

The function of phagocytic cells of the immune system is known to degrade in age. Also, the incidence of autoimmunity in age might result from non-removed debris [65].

Microglia vs Macrophages: Concerning surface markers, primary microglia and macrophages differ only in their levels of expression. Microglia have been reported to express cluster of differentiation 11b (CD11b) like macrophages but have only low CD45 while macropages show high CD45 levels [66, 67]. Also, low CD68 was observed in microglia and high CD68 in macrophages [68]. Furthermore, they differ in substance P levels [4]. These different levels of expression correlate with activation states. Activated microglia are virtually indistinguishable from macrophages derived from monocytes [49]. Also, the overlap in markers between dendritic cells, macrophages and microglia has prompted a discussion about their distinction. Peripheral blood monocytes and bone marrow can be differentiated to microglia using astrocyte-conditioned medium (ACM). ACM not only differentiates microglia but also lowers functions like phagocytosis, oxidative burst and migration of microglia. It also causes the typical ramification phenotype and the growth of the processes visible in ramification. If supplemented with ACM, macrophages show this ramification.

Usually, macrophages are differentiated and expanded using M-CSF [12, 38]. On the other hand, ramified microglia can be activated and phenotypically reverted to amoeboid or round cells resembling macrophages or monocytes by addition of LPS [4].

It has been proposed that microglia could be immature macrophages that mature if exposed to activating signals [38]. Another line of thought suspects that microglia are down-regulated, resting macrophages. This is mainly founded on the correlation of marker expression and activation states and on the reversibility of activation by cytokine supplementation.

Adult microglia might possess distinct electrophysiological characteristics that differ from that of macrophages [69]. These characteristics are also present on a subset of bone marrow resident progenitors (Ibid.).

Furthermore, there are indications that tissue resident macrophages are distinct from adult macrophages [70]. It has been proposed that the subpopulation of tissue resident macrophages arise from progenitors prior to vascularization of organs. In the adult, macrophages arise mainly from the bone marrow as the major hematopoietic tissue.

2. Manuscript Overview

Paper 1: “Differentiation of Mouse Bone Marrow derived Stem Cells towards Microglia-like Cells.”

Authors: Arnd Hinze, Alexandra Stolzing, Published

Hinze, AH, Stolzing, A: **Differentiation of Mouse Bone Marrow derived Stem Cells towards Microglia-like Cells.** *BMC Cell Biology* 2011, **12**:35

Several protocols for the differentiation of microglia were screened. The differentiated cells were compared for marker expression, function (phagocytosis, oxidative burst), morphology and migration behaviour. The resulting best protocols are described in the publication. Also, the influence of certain cytokines (Flt3L, GM-CSF), time and density on the differentiation were investigated.

Arnd Hinze carried out all experiments and wrote the manuscript. Alexandra Stolzing designed and coordinated the study and contributed to writing the manuscript.

Paper 2: “Microglia differentiation using a culture system for the expansion of mice non-adherent bone marrow stem cells.“

Authors: Arnd Hinze, Alexandra Stolzing, Published

Hinze, AH, Stolzing, A: **Microglia differentiation using a culture system for the expansion of mice non-adherent bone marrow stem cells.** *Journal of Inflammation* 2012, **9**:12

Non-adherent bone marrow cells are suspected to correspond to a circulating cell population. They might, therefore, harbor microglia progenitor cells. The cell culture of non-adherent bone marrow cells is further characterized in the paper and their differentiation capacity to microglia was tested.

Arnd Hinze carried out all experiments and wrote the manuscript. Alexandra Stolzing designed and coordinated the study and contributed to writing the manuscript.

Paper 3: "To Migrate or not to Migrate – Microglia and Cell Migration to and from the Brain."

Authors: Arnd Hinze, Alexandra Stolzing, Submitted to Journal of Inflammation, 10/2012

Migration of cells into the brain is subject to many conditions, age and diseases. The review summarizes conditions under which cells migrate across the BBB and into the brain. It also delves into conditions that influence the success of cell therapies directed at the brain.

Arnd Hinze designed and wrote the manuscript. Alexandra Stolzing contributed to writing the manuscript.

3. Paper 1: “Differentiation of Mouse Bone Marrow derived Stem Cells towards Microglia-like Cells.”

Differentiation of mouse bone marrow derived stem cells toward microglia-like cells

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Running title: Microglia from stem cells

Key words: bone marrow stem cells, microglia, Flt3L, GM-CSF, neurodegeneration, differentiation

Abstract

Background: Microglia, the macrophages of the brain, have been implicated in the causes of neurodegenerative diseases and display a loss of function during age. Also, recent studies emphasized the heterogeneity of microglia in the brain and linked it to the potential of microglia to induce tolerance or immune reaction. It is therefore of great interest to investigate the differentiation of microglia for development of actual immune function. We differentiated here microglia from bone marrow stem cells (BM). Furthermore we looked at the effects of FMS-like tyrosine-kinase-3 ligand (Flt3L), astrocyte conditioned medium (ACM) and GM-CSF on the differentiation to microglia-like cells.

Methods: We assessed *in vitro* derived microglia differentiation by marker expression (CD11b/CD45, F4/80), but also for the first time for functional performance (phagocytosis, oxidative burst) and in situ migration into living brain tissue. Integration, survival and migration were assessed in organotypic brain slices.

Results: The cells differentiated from mouse BM show function, markers and morphology of primary microglia and they migrate into living brain tissue. Flt3L displays a negative effect on differentiation while GM-CSF enhances differentiation.

Conclusion: We conclude that *in vitro* derived microglia are the phenotypical and functional equivalents to primary microglia and could be used in a cell therapy.

Background

Microglia comprise about 10% of the cell population of the brain and form the main first immune defense of the CNS. They are phagocytic, cytotoxic, present antigens and promote repair after injury [1]. Primary microglia differ from other blood macrophages in the expression levels of markers like CD11b/CD45low/high [2], CD68 low/high [3] and substance P levels [4]. Because of the overlap in markers there is an ongoing discussion about the distinction between dendritic cells, macrophages and microglia. The microglia cell population partially shows dendritic cell phenotypes [5]. This heterogeneity has been linked to activation states of microglia and the potential to induce tolerance or immune reactions [6, 7]. It is therefore of great interest to investigate the differentiation of microglia from bone marrow – especially in view of possible subpopulations acting tolerance inducing which might be identified by expression of antigens implied in tolerance [6] and by tests of immune function *in vitro*.

Microglia in the brain display normally a quiescent state in which phagocytosis, immune response and migration are down-regulated and the microglia show a ramified form with long processes [8]. Microglia react to inflammation by turning to an activated state and taking on an amoeboid morphology [9]. They migrate towards injury, lesions and extracellular debris such as amyloid-β plaques [10]. An important function of microglia is the "oxidative burst" – a sudden spike in reactive oxygen species (ROS) levels generated by the stimulation of the NADPH oxidase. This ROS production is accompanied by the release of other factors, including lysosomal proteases. This mechanism, often interpreted as a ‘defense’ response that can protect the brain from pathogens is a typical feature of microglia [11, 12].

Microglia are thought to originate from the yolc sac during embryogenesis [13]. They are thought to be replenished during life mainly by local proliferation. The replenishment by progenitor cells from the bone marrow is controversial [1, 13, 14]. Bone marrow derived microglia can be observed in the brain after systemic transplantation [15]. While BM chimeras have shown BM derived microglia [16], other findings indicate that without irradiation no invasion is observable in the time frame of 1-2 months [17, 18]. But also in transplantations without irradiation intravenously injected hematopoietic stem cells have been observed to migrate to the brain, differentiate into microglia and reduce infarct size [19].

The maturation of progenitors to microglia occurs under the influence of factors secreted by astrocytes [20]. Both local and peripheral replenishment seem not to suffice to prevent the slow deterioration of the microglia cell population and function in age [21, 22]. In human Alzheimer patients microglia associated with tau were found to be fragmented and this dystrophy might preceed neurodegeneration [23]. In old rats there have been indications that proliferation of microglia after injury is stronger than in young rats [24]. *In vitro*, microglia have been reported to undergo telomere shortening [25] and aged microglia have been observed to loose their ability to perform normal microglia functions [21, 22, 26-30]. All of these findings support the hypothesis of a slow deterioration of microglia as a contribution to the onset of neurodegeneration [22, 23]. Observations that amyloid beta plaques were not colocalized with activated microglia might indicate that they are not responsible for a chronic activation [23]. Also, anti-inflammatory drugs have not shown a clear enhancement of Alzheimer condition [31, 32]. Therefore, chronic activation might be a later occurring step in neurodegenerative diseases.

Microglia have been differentiated *in vitro* from peripheral blood monocytes [4, 20] and from embryonic stem cells [33]. Here, we are focusing on differentiating microglia from bone marrow. This approach was first demonstrated by [34] who obtained a 20% occurrence in microglia-like morphology and marker expression (CD115+, CD11b+, F4/80+, CD80 low, CD86-) after culturing mouse BM cells in Flt3L for 11 days and then treating the supernatant with astrocyte conditioned medium for 6 days. However, since the use of Flt3L was not controlled in that protocol, its role as a factor in microglia differentiation remained unclear.

Davoust et al. [35] used a similar protocol but much shorter culture times, and no Flt3L to obtain CD11b +, CD45 + , MHCII - , B220 low, CD34+, CD86 low cells from mouse BM (the percentage yield is not given). The success of microglia cell differentiation has been mostly judged by measurement of marker expression and morphology of the differentiated cells. It was unclear to what extent *in vitro* derived microglia share the functional capacities of microglia in vivo. To address this question, we followed the protocol of Servet-Delprat et al. [34] (with and without Flt3L), measured phagocytosis and oxidative burst as hallmarks of microglia function and tested the ability to survive and migrate in brain tissue.

Results

Surface marker expression

Untreated bone marrow cells showed significantly increased CD11b/CD45 expression after 17 days. The same was seen in cultures treated with ACM/GM-CSF. Non adherent BM cells treated with ACM/GM-CSF and whole bone marrow cultivated for 17 days are observed in the same region in the flow cytometry plots as primary microglia. Flt3L has an adverse effect on differentiation, leading to low levels of CD11b/CD45 positive cells in all Flt3L supplemented samples.

The frequency of F4/80+ cells increased significantly in whole bone marrow already after 7 days and also after 17 days compared to fresh bone marrow. The supplementation of Flt3L or Flt3L/ACM/GM-CSF resulted in significantly lower F4/80+ cell numbers while the addition of only ACM/GM-CSF yielded high numbers of F4/80+ cells very similar to the CD11b/CD45 cell populations (Fig. 1, table 1).

Time course of marker expression

The frequency of CD11b+/CD45+ and F4/80+ cells in whole bone marrow rose steadily and significantly with culture time (Fig. 2, table 1). The additional supplementation of ACM/GMCSF to the non-adherent BM cells increased CD11b+/CD45+ and F4/80+ cells over time, however the increase was significantly slower and only reached the same level as un-supplemented bone marrow at day17. The supplementation of Flt3L resulted in a lower frequency of CD11b+/CD45+ and also F4/80+ (Fig. 2, table 1). The addition of ACM/GMCSF increased the medians of CD11b expression significantly after 10 days (Fig. 2, table 1).

Phagocytic activity and oxidative burst

Whole bone marrow cultured over a period of 7, 10 or 17 days showed a constant significant higher percentage of phagocytic cells compared to fresh bone marrow (Fig. 3A). Supplementation with ACM/GM-CSF increased the number of phagocytotic cells significantly compared to un-supplemented bone marrow at day 17. Flt3L supplementation inhibited the differentiation towards phagocyting microglia significantly even when ACM/GM-CSF was added. We observed the same changes in the amount of microglia performing oxidative burst (Fig.3b), however the differences were less pronounced.

Cell morphology

Primary microglia show long processes and rod shaped cells (Fig. 4A). Un-supplemented bone marrow cells had mixed morphologies during the whole cultivation time (Fig. 4B-E). The cells supplemented with ACM/GM-CSF are homogenous and show strong ramification (Fig. 4G). Cells treated with Flt3L alone or in presence of Flt3L and ACM/GM-CSF both have a more fibroblastic morphology with no resemblance to microglia (Fig. 4H, 4I).

Migration in organotypic brain slices

Whole brain slices were cultured for 10 days to minimize surface damage before differentiated microglia pre-labeled with 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) were added on top of the brain slices. The slice was counterstained with propidium iodide (PI) to visualize dead cells. Reconstructed confocal pictures were either top-down or showing a side view of the slice. Microglia were observed over a period of 10 days and found to survive and proliferate (Fig. 5A-C, dead cells deliberately included for reference). Over the course of 3 days ACM/GM-CSF supplemented BMC migrated into the surface of the brain slices as deep as 50µm (Fig. 5D-F). Cells of all protocols migrated up to 30µm into the slice after 10 days, while dead cells stayed on top of the tissue (Fig. 6A, 6B, 6C, 6D). Several cells migrated up to 120µm (scan depth of confocal microscope was 160µm). Cells supplemented with Flt3L have the same fibroblastic morphology as in the *in vitro* cultures (Fig. 6B) while additional supplementation with ACM/GM-CSF resulted in round and amoeboid cells as well as fibroblastic cells (Fig. 6C). Cells supplemented only with ACM/GM-CSF showed almost exclusively round cell morphology (Fig. 6D). Cells of whole bone marrow were round but bigger than the ACM/GM-CSF supplemented cells after 7 days and did not migrate more than 30µm into the surface of the brain tissue (Fig. 6A).

Discussion

We investigated here the differentiation and function of microglia from bone marrow (BM) stem cells using ACM and GM-CSF with and without Flt3L. We used GM-CSF as opposed to M-CSF used by Davoust et al. [35] as this is reported to expand primary microglia more successfully than M-CSF [40, 41]. Primary microglia have been characterized as CD11b+/CD45low and have been distinguished from primary macrophages by their CD45 expression level [2]. The *in vitro* differentiated microglia derived here generally show similar marker expression levels as primary microglia. It is known that ACM treatment of BM cells can produce cells with markers for microglia [34].

However, such cells have not been further characterized with regards to phagocytotic capacity, migration behavior inside the brain or tested for the microglia-typical oxidative burst. Here we demonstrate that BMC cultured in the presence of ACM, GM-CSF show microglia typical phagocytosis and oxidative burst activity. The cells also had long and branched processes similar to primary microglia. Flt3L supplementation diminished the functional markers and microglia like morphology. Thus, among the parameters tested here, the ‘optimal’ protocol for *in vitro* differentiation of microglia relies on ACM, GM-CSF without Flt3L.

Interestingly, we find that even un-supplemented BM contains a subpopulation positive for microglia markers (CD11b/CD45, F4/80) and that this population is more dominant after 17 days differentiation. However, we find that microglia-like cells derived from BM without any supplementation only display low phagocytosis and oxidative burst levels compared to ACM/GM-CSF supplemented cells.

Generally, un-supplemented bone marrow cultures show mixed cell morphologies whereas supplemented cultures are more likely to display homogeneous branched morphologies.

Flt3L has been used for the sequential differentiation of BM cells presumably because it improves hematopoietic stem cell (HSC) survival *in vitro* [34] and *in vivo* [42]. Servet-Delprat et al. only looked at Fl3L supplemented cells and did not consider un-supplemented cells. The group estimated 20% microglia from the number of ramified cells which is confirmed by our results for ACM, GM-CSF, Flt3L supplemented cells. However, much higher microglia ‘yield’ can be obtained in the absence of Flt3L. In fact, we demonstrate that supplementation with Flt3L diminishes microglia differentiation: where Flt3L is added by itself of in combination with ACM, GM-CSF, the number of cells showing microglia markers, as well as the capacity for brain migration, phacocytosis and oxidative burst decreases.

The differentiation protocols investigated here rely on using the supernatant at day 11 to select for non-adherent HSC which is then cultured in the presence of ACM for another 6 days. The tactic here is to first obtain a relatively pure HSC population which then differentiates in part into adherent microglia. Flt3L has been shown to expand HSC, transiently increase adhesion of HSC in culture and might play a role in mobilization of HSC to the blood stream [43] (Supplementary figure 2). Therefore the amount of microglia progenitor cells in the supernatant of the 11 day bone marrow culture might be decreased or the differentiation might be delayed. In addition Flt3L combined with GM-CSF has been shown to enhance dendritic cell differentiation [44]. This fact is supported by work with Flt3L knockout mice where levels of dendritic cells are increased and numbers of myloid cells, the progenitors for microglia, are decreased [45]. These factors may explain why Flt3L supplementation yields a lower count in functional *in vitro*-derived microglia.

The microglia cell population is known to be heterogeneous and to overlap with dendritic cell like populations in the brain [46]. The various procedures employed for microglia differentiation might result in distinct subpopulations or activation states. The choice of the protocol might have a large impact on the effect transplanted cells will have *in vivo*. This is especially important because different subsets of microglia have been linked to induction of tolerance or immune reaction [6].

The expression of Tmem176b and TREM2 has been associated with tolerance of microglia [6]. Also, the loss of function of certain receptors like the triggering receptor (TREM2) or DNAX-activating protein 12kDa (DAP12) has been observed to lead to an inflammatory neurodegenerative disease later in life [47]. Differentiated microglia carrying such markers could be beneficial to reduce reaction to transplants [48] or to treat autoimmune inflammation, for example in acute experimental autoimmune encephalomyelitis [5, 49]. Transplantation of human microglia in ischemic brains modulated inflammation and reduced neuronal apoptosis [50]. Microglia provided neuroprotection in hippocampal slice cultures while lipopolysaccharide-stimulated microglia did not [51].

However, differentiation of immunogenic dendritic like cells from bone marrow might result in immune reaction if used in transplantation. In the current study, microglia were differentiated using ACM and GM-CSF. There is evidence that cells showing an immature dendritic phenotype can differentiate from microglia under the influence of GM-CSF [46]. At the same time, dendritic cells can be differentiated to microglia like cells which inhibit T cell proliferation induced by mature dendritic cells [52]. Dendritic cells can act both tolerogenic or immunogenic, depending on their maturation state [48].

In co-cultures with organotypic brain slices the differentiated microglia survived and proliferated for at least 10 days. It is known that the majority of primary microglia or BV2 cells just migrate over the surface layer of brain tissues under non-inflammatory conditions [53, 54] while a subpopulation migrates into the tissue. Directed migration towards sites of injury induced by NMDA on the surface of brain slice cultures has been observed for primary microglia [54]. The damaged surface of the brain slice cultures even attracts slice internal microglia which showed directed migration to the surface [53]. This is supported by our results: Most cells migrate into the brain slice tissue superficially, while *in vitro* derived (ACM/GM-CSF, but without Flt3L) microglia migrated deepest into the tissue and showed both amoeboid and rounded morphologies suggesting an activated state.

Conclusion

The *in vitro* differentiated cells correspond in phenotype and function to primary microglia. If neurodegenerative diseases occur in part due to a deterioration of the microglia cell population or function with age, functional microglia supplementation could have beneficial effects. For example, injection of primary microglia into the brain of rats led to an increased amyloid beta clearance [55] Furthermore, the suspected ability of microglia precursors to cross the blood brain barrier and to seek out sites of neuroinflamation makes them potentially useful drug delivery vehicles [56]. *In vitro* derived microglia will need to demonstrate the functional capacity of ‘real’ microglia cells and this research makes some contributions towards this aim. Also, the differentiated microglia could now be screened for markers of tolerance inducing microglia subsets and for the actual induction of tolerance *in vitro* [6].

Methods

Animals

C57BL/6 mice from the MEZ of the University of Leipzig and Charles River (Sulzfeld, Germany) were used as sources for bone marrow, primary microglia and organotypic brain slices in accordance with local animal ethics permissions.

Isolation of bone marrow and cell culture

Bone marrow was obtained by centrifugation of femurae and tibiae. Isolated bone marrow cells were cultured at a density of 10^7 cells in a 60mm petri dish in 5ml of Dulbecco minimal essential medium (DMEM)/low glucose (Hyclone Laboratories Inc.) supplemented 10% fetal calf serum (FCS -Invitrogen) and 100 units/ml Penicillin, 100 µg/ml Streptomycin.

Astrocyte conditioned medium

Astrocyte conditioned medium was produced by incubating medium (DMEM/10% FCS) 24h with primary astrocyte cultures [16].

Isolation of primary microglia

Primary microglia were isolated from brains of 1-3 day old mice. The meninges was removed and the whole brain was titurated in DMEM/10% FCS and Pen/Strep. The resulting cell and tissue suspension of 3 brains was cultured in a poly L-lysine coated culture flask. After 24h the supernatant was removed from the cell culture and new medium added. After 7 days 50% of culture medium was changed. At 14 days microglia were removed by gentle shaking [36].

Differentiation towards microglia-like cells

Whole BMC: Whole bone marrow (10^7 cells) was cultivated over the time periods of 7, 10 and 17 days in 10ml DMEM/10% FCS in a 60mm petri dish. When cells were cultured for longer than 10 days 50% of medium was replaced at day 10 (Supplementary figure 1). BMC + ACM/GM-CSF: 107 cells were cultured for 11 days in a 90mm petri dish in DMEM/10%

FCS. After 11 days, non adherent cells from 2 petri dishes were flushed off. These cells were transferred to a new 60mm petri dish and cultured for additional 6 days in DMEM/10% FCS, 50% ACM, 20ng/ml GM-CSF (Supplementary figure 1). BMC + Flt3L/ACM/GM-CSF: 107 cells were cultured for 11 days in a 90mm petri dish in DMEM/10% FCS/5ng/ml Flt3L. The non adherent cells of day 11 were transferred to a 60mm petri dish. Afterwards the cells were cultured in DMEM/10% FCS, 50% ACM, 20ng/ml GMCSF for 6 days. BMC + Flt3L: 10^7 cells were cultured for 11 days in a 90mm petri dish in DMEM/10% FCS/5ng/ml Flt3L. After transferring the non adherent cells of day 11 were to a 60mm petri dish they were cultured in DMEM/10% FCS for 6 days (Supplementary figure 1).

Flow cytometry

The differentiated cells were tested for the markers F4/80, CD11b, CD45 and CD11b/CD45 double expression. The cells were trypsinized, centrifuged at 300g for 5min and fixed in 4% paraformaldehyde. They were washed with phosphate buffered saline (PBS). Afterwards cells were incubated for 2h at 4°C with CD11b (1:250) or F4/80 (1:250) antibody (both Alexa 488 labeled, eBioscience) or with CD45 (1:100) antibody (PE labeled, eBioscience). The incubated cells were washed again and fluorescence measured with a Beckmann Coulter FC 500.

Phagocytosis

Phagocytic activity of the differentiated cells was measured by the uptake of fluorescent beads (Sigma, 2µm yellow green fluorescent). In a first step samples of $3*10^5$ cells were activated with phorbol myristic acid (PMA) (0.1µM) for 15min at 37°C [37]. Afterwards they were incubated in 50µl DMEM/10% FCS together with 50µl opsonized (FCS) beads for 48h at 37°C, 5% CO_2. The uptake of fluorescent beads was observed qualitatively in a Zeiss Axio Observer fluorescence microscope. For quantitative assessment the cells were trypsinized and resuspended in PBS (Invitrogen). Cells were repeatedly washed and fluorescence was measured in a Beckmann Coulter FC 500.

Oxidative burst

Nitro Blue Tetrazolium (NBT): 10^4 cells were seeded on cover slips. They were incubated with 30µl 1mg/ml NBT and 100 nM PMA for 45 min at 37°C and 5% CO_2 [38]. Light microscope pictures were taken with a Leica DM IL (Leica) using the LAZ EZ 1.4.0 software (Leica). Pictures were brightness and contrast adjusted with GIMP 2.4.5 and power point (Microsoft).

Dihydrorhodamine 123 (DHR123): $3*10^5$ Cells were incubated in PBS for 15min at 37°C with 0.1µM PMA. Controls were incubated without PMA. Afterwards 50µM DHR123 (Invitrogen) was added and the cells were incubated for additional 15min at 37°C. The cells were fixed with 4% PFA and fluorescence was measured in a Beckmann Coulter FC 500.

Cell Morphology

A DM IL (Leica) and the LAZ EZ 1.4.0 software (Leica) was used to take light microscopic pictures.

Brain slice cultures

2-3 month old C57BL/6 mice were killed by cervical dislocation. The brain was isolated and cut with a Leica VT 1000 S vibratome in 350µm slices in cold preparation medium (HBSS and 10% FCS (Invitrogen)). The slices were transferred to an insert (Millicell CM 0.4 µm, Millipore) and cultivated with brain slice culture medium (50% DMEM/high glucose (HyClone Laboratories Inc.), 25% Horse Serum (Invitrogen), 25% HBSS (Invitrogen), 1 µg/ml insulin, 100 units/ml penicillin, 100 µg/ml streptomycin). Medium was changed every 2-3 days. The brain slices were cultured for 9 days before cells were seeded on them [39].

Survival and migration in brain slice cultures

Differentiated cells were labeled for 20min with DIO (Invitrogen). They were added to the top of the brain slices. The viability of brain slice cultures was checked by performing PI staining. The survival of seeded cells was checked by adding 5µg/ml propidium iodide in the medium, washing and scanning the slices with a confocal microscope TCS SP2 (Leica Microsystems) using the accompanying software LCS 2.6 (Leica Microsystems). The slices were scanned after 10 days of co-culture to a depth of 160 µm. Images were contrast and brightness adjusted with GIMP 2.4.5 and power point (Microsoft).

Statistical Analysis

All data are presented as means ± SE. Statistic analysis were made using SigmaPlot 10.0/SigmaStat 3.5 software (SYSTAT, Erkrath, Germany).

Authors' contributions

Arnd Hinze carried out all experiments and wrote the manuscript. Alexandra Stolzing designed & coordninated the study and wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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Figure legends

Figure 1: **(A)** Flow cytometric analysis of differentiated cells concerning their CD11b/CD45 expression (n=3). Representative scatter plots of CD11b/CD45 labeled differentiated cells and primary microglia, fresh bone marrow and an isotype control.

(B) Flow cytometric analysis of differentiated cells concerning their F4/80 expression (n=3). Representative histogram plots of F4/80 labeled differentiated cells and primary microglia, fresh bone marrow. Isotype control gray, F4/80 labeled cells black. *** = P<0.001, ** = P<0.01, * = P<0.05.

Figure 2: Time course of CD11b/CD45 and F4/80 expression of untreated and cytokine treated BMC (n=3). Time course of CD11b and F4/80 medians. Significant changes are denoted with respect to freshly isolated BMC on day 0. *** = P<0.001, ** = P<0.01, * = P<0.05.

Figure 3: **(A)** Phagocytosis of differentiated cells and fluorescence microscope picture of phagocytosis of non adherent BM cells differentiated with ACM/GM-CSF (n=3). Arrows indicate the same cell in bright field and fluorescence picture. Fluorescence images were taken with a Zeiss Axio Observer at 200x (left) and 400x (right) magnification.

(B) Oxidative burst of differentiated cells, representative histogram plot of inactive (open histogram) and PMA activated (red histogram) cells (n=3). The shift between un-treated and PMA treated microglia was measured as quotient between medians of treated and untreated cells (Fluorescence signal noise ratio – FSN). Cells with no shift are based at 1 and higher numbers represent cell populations which did show ROS production. Light microscope picture of NBT reduction of non adherent BM cells supplemented with ACM/GM-CSF. Arrows indicate one cell with dark blue NBT precipitate and one cell without precipitate. The picture was taken with a Leica DM IL at 20x magnification. *** = $P<0.001$, ** = $P<0.01$, * = $P<0.05$.

Figure 4: Representative light microscope pictures of differentiated cells. Images were taken with a Leica DM IL at 200x magnification. BMC: Whole bone marrow cells. Fluorescence picture of an Iba-1 stained microglia in a brain slice **(B)** was taken with an Axio Imager A1 (Zeiss) at 63x magnification.

Figure 5: Coculture with living brain slices. Differentiated ACM/GM-CSF treated BMC were labeled with DIO and seeded on brain slices on day 9. Counterstaining with propidium iodide was used to assess cell survival. After 1, 2 , 3, 6 and 10 days slices were scanned with a Leica Microsystems TCS SP2 confocal microscope to assess survival of seeded cells and their migration into the tissue. Arrows indicate single cells that have already migrated through the surface after 2 days. Magnification was 100x and scanning depth was 160 µm.

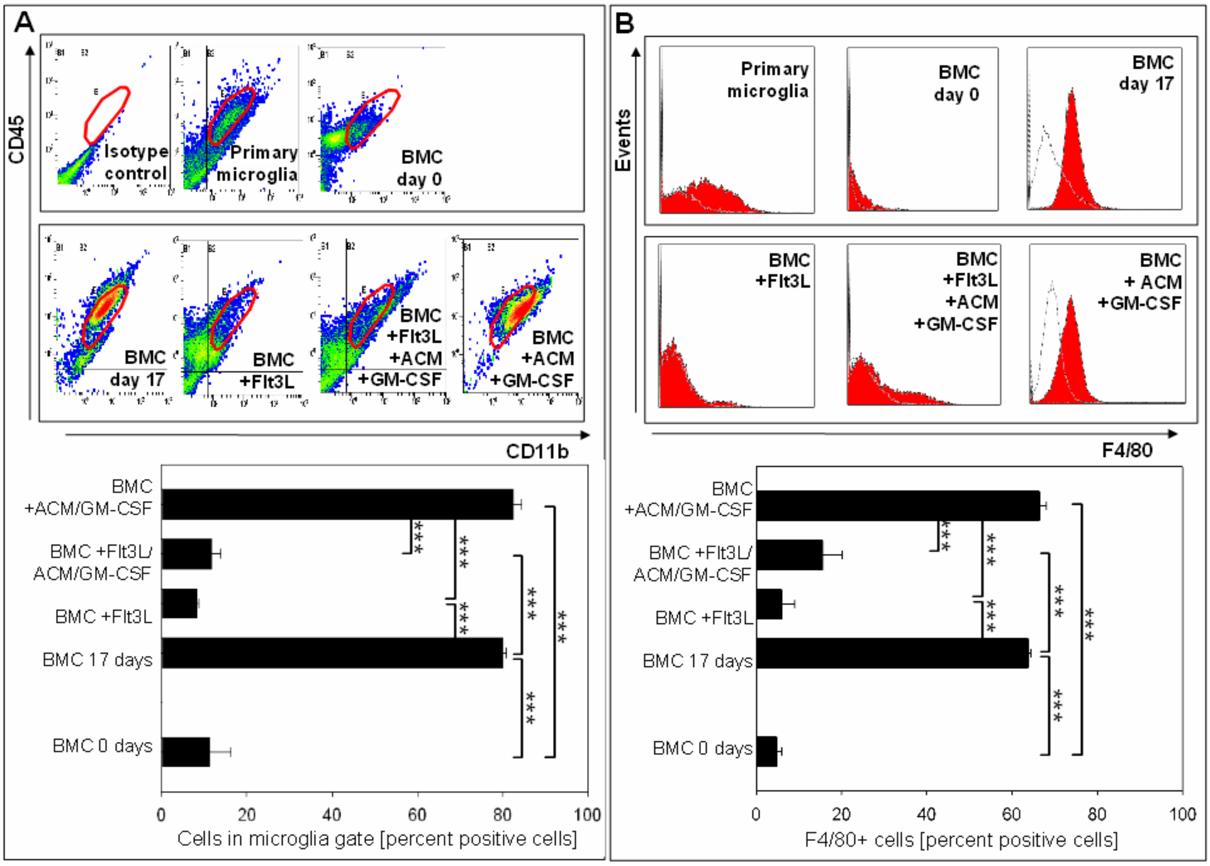
Figure 6: Differentiated cells of all protocols were labeled with DIO and seeded on brain slices on day 9. After 10 days coculture slices were propidium iodide stained and scanned with a Leica Microsystems TCS SP2 confocal microscope to measure cell survival and their migration. Arrows indicate several cells that have migrated deeper into the brain tissue. The images were taken at 100x magnification and the brain slices were scanned to a depth of 160 µm.

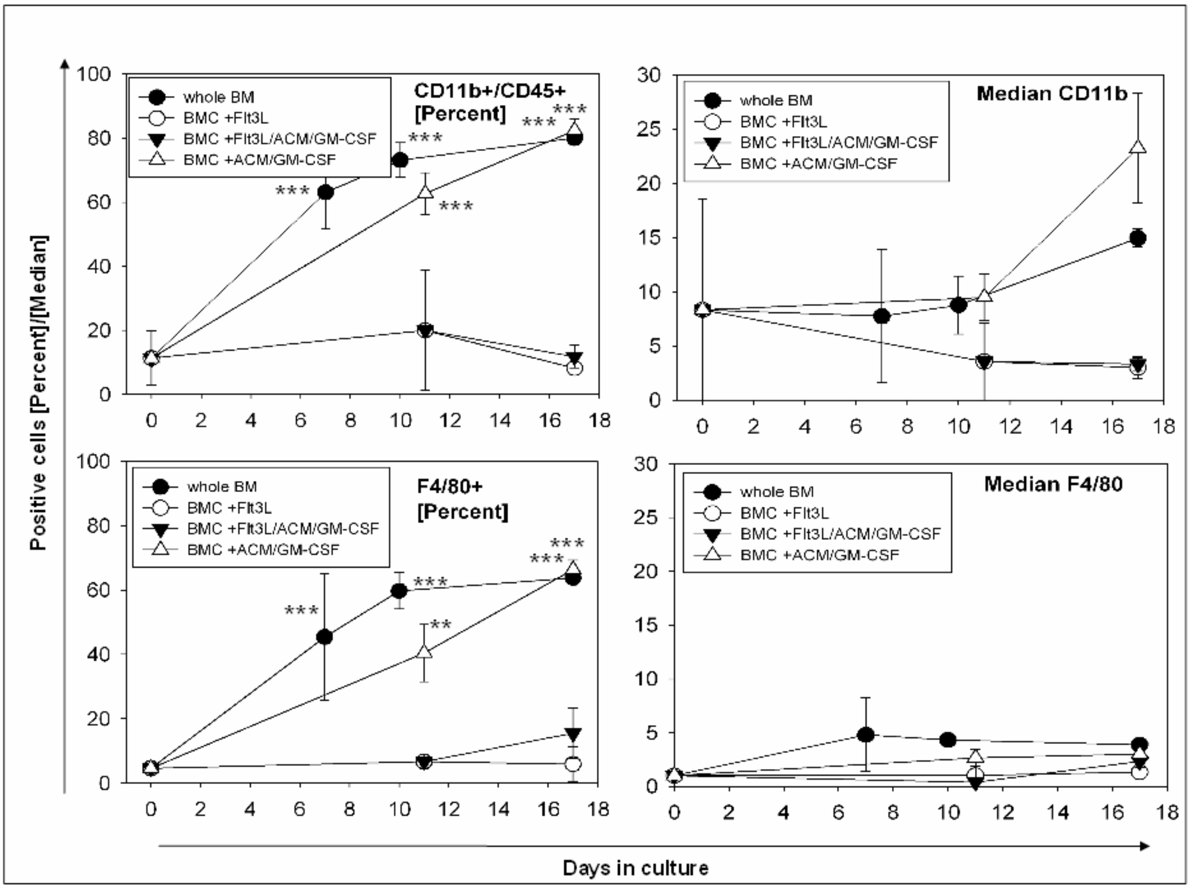
Tables

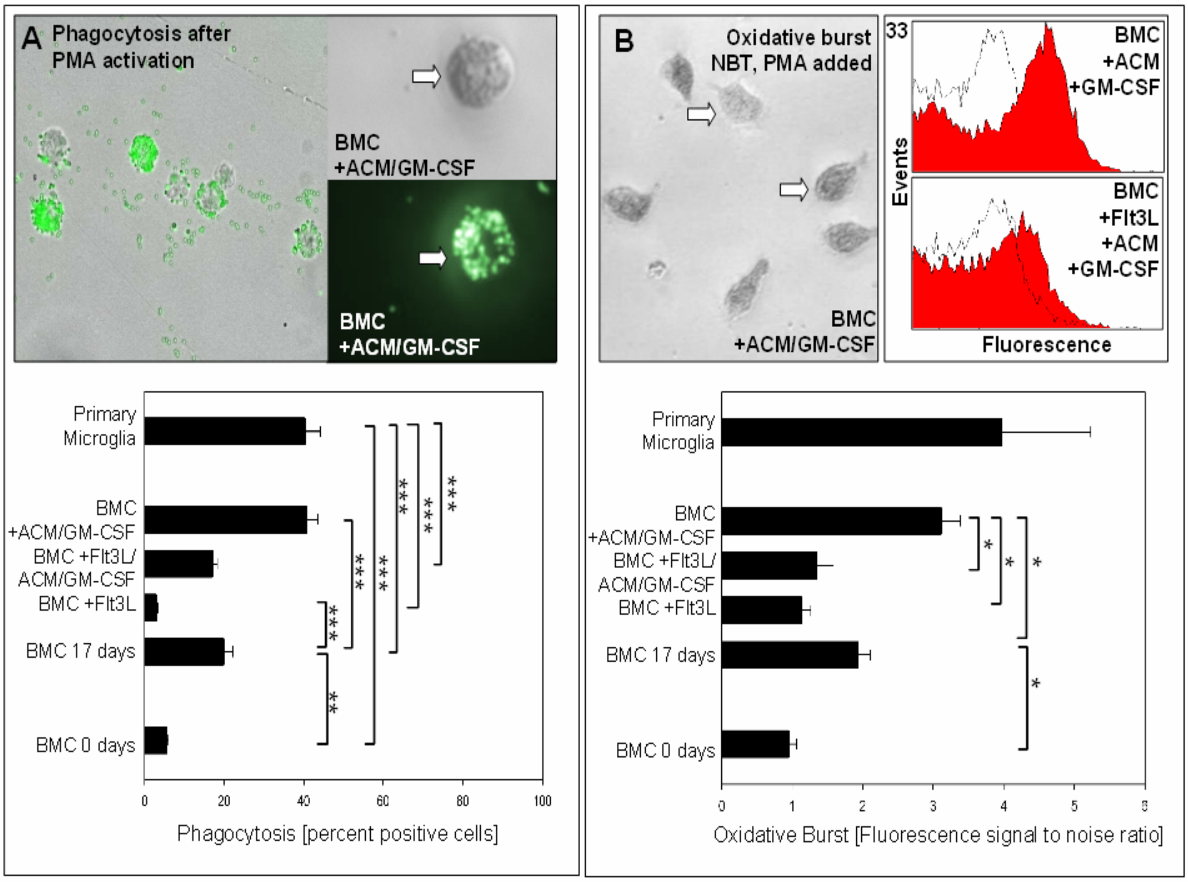
Table 1: Flow cytometric analysis of the cells differentiated and analyzed for microglia specific markers.

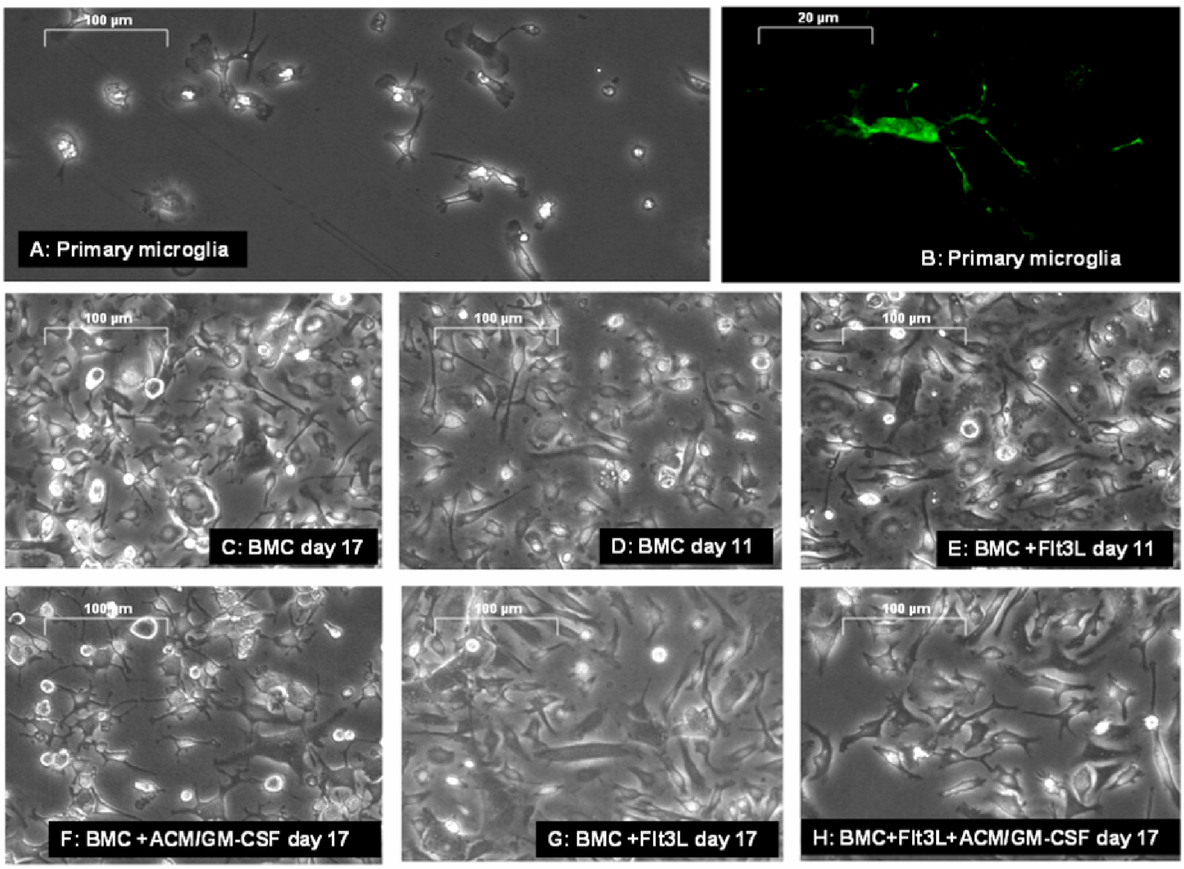
Protocol	**Supplementation**	**CD11b percent**	**CD11b median**	**F4/80 percent**	**F4/80 median**	**CD11b/CD45 percent**	**Cells in microglia gate**
BM 0 days		**20** +/-9.5	**2** +/-0.7	**2.3** +/-0.1	**1.2** +/-0.1	**20** +/-9.5	**6,7** +/-3,6
BM 7 days		**51,2** +/-15,5	**7,8** +/-3,5	**55,9** +/-5,3	**4,4** +/-0,6	**54,7** +/-15,0	**54** +/-19,7
BM 10 days		**67,3** +/-2,8	**8,8** +/-1,5	**59,8** +/-3,3	**4,3** +/-0,3	**72,8** +/-2,9	**70,3** +/-4,1
BM 17 days		**68,4** +/-0,6	**13,8** +/-0,4	**64,5** +-0,6	**3,6** +/-0,1	**70** +/-0,5	**84,2** +/-0,5
Supernatant of BM culture on day 11		**71,7** +/-3,6	**9,5** +/-1,3	**40,5** +/-5,1	**2,7** +/-0,4	**71,7** +/-3,6	**62,7** +/-6,5
Supernatant of BM culture on day 11	Flt3L	**35,8** +/-12,9	**3,6** +/-2,1	**6,8** +/-1,3%	**1** +/-0,2	**35,8** +/-12,9	**20,1** +/-18,8
Protocol 2	ACM/GM-CSF	**85,6** +/-1,5	**23,3** +/-2,9	**66,4** +/-1,7	**3** +/-0,3	**89,1** +/-1,5	**90** +/-1,7

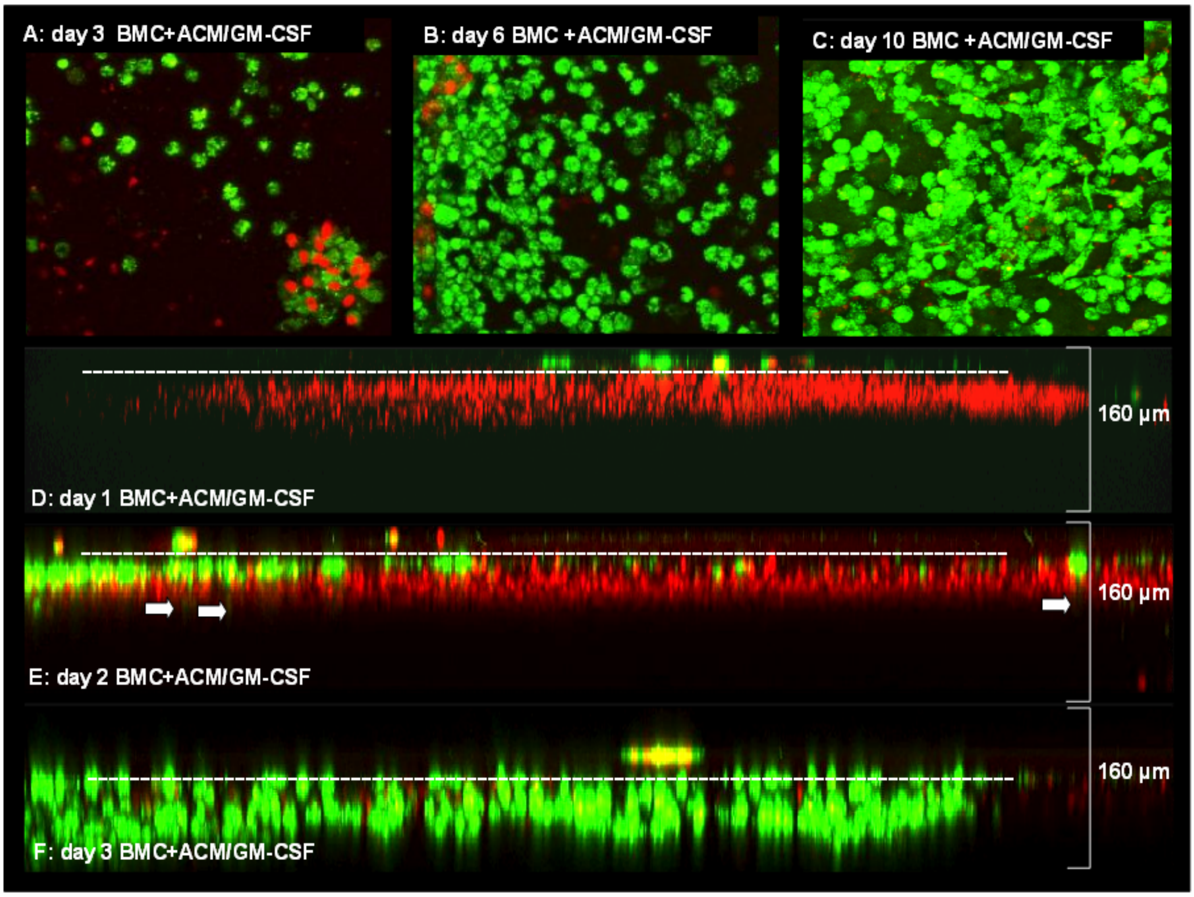
Protocol 3	Flt3L/ACM/GM-CSF	**39,1** +/-8,1	**2** +/-0,3	**18,8** +/-3,1	**1,3** +/-0,2	**46,6** +/-8,0	**9,9** +/-2,7
Protocol 4	Flt3L	**8,2** +/-4,1	**1** +/-0,0	**10,7** +/-5,1	**1,1** +/-0,1	**15,5** +/-4,1	**5,1** +/-0,5

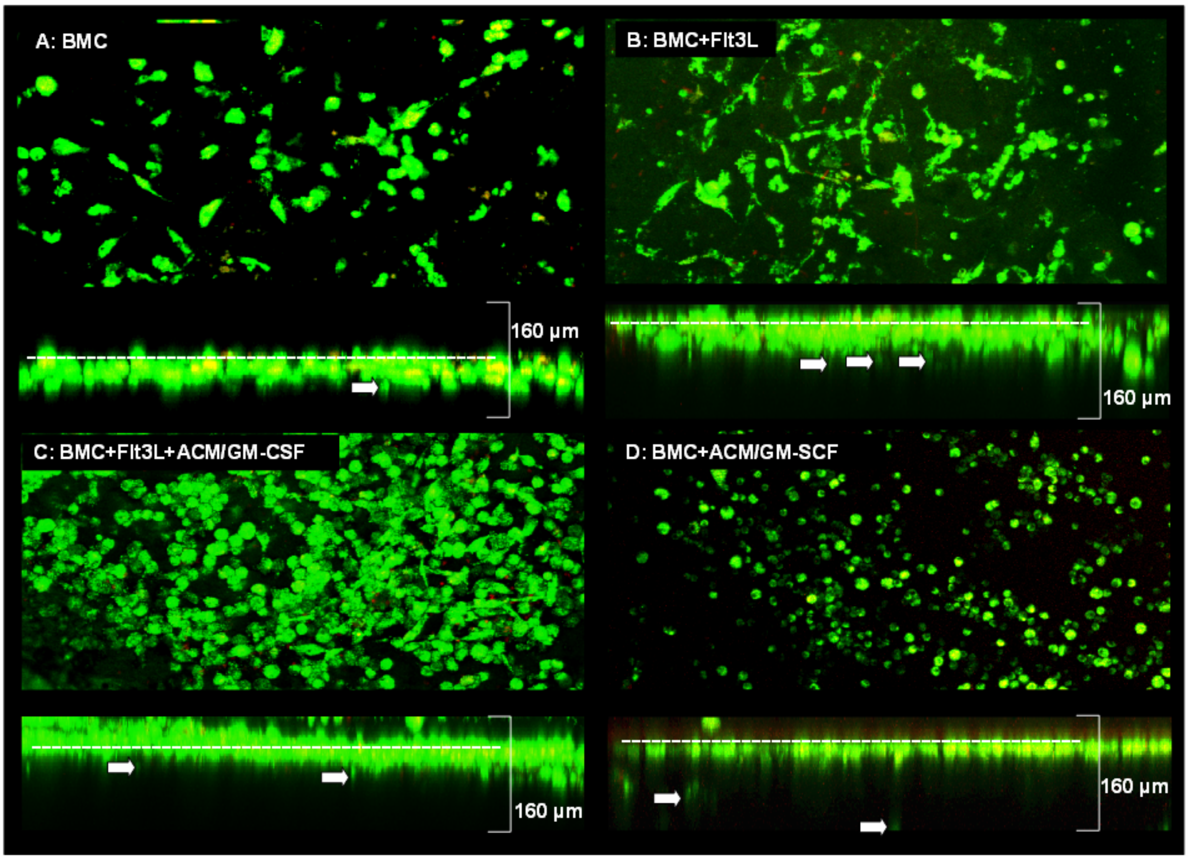

A
CD45
Isotype control
Primary microglia
BMC day 0
BMC day 17
BMC +Flt3L
BMC +Flt3L +ACM +GM-CSF
BMC +ACM +GM-CSF
CD11b
BMC +ACM/GM-CSF
BMC +Flt3L/ ACM/GM-CSF
BMC +Flt3L
BMC 17 days
BMC 0 days
Cells in microglia gate [percent positive cells]
B
Events
Primary microglia
BMC day 0
BMC day 17
BMC +Flt3L
BMC +Flt3L +ACM +GM-CSF
BMC + ACM +GM-CSF
F4/80
F4/80+ cells [percent positive cells]


Positive cells [Percent]/[Median]
whole BM
BMC +Flt3L
BMC +Flt3L/ACM/GM-CSF
BMC +ACM/GM-CSF
CD11b+/CD45+ [Percent]
Median CD11b
F4/80+ [Percent]
Median F4/80
Days in culture


A Phagocytosis after PMA activation
BMC +ACM/GM-CSF
BMC +ACM/GM-CSF
B Oxidative burst NBT, PMA added
BMC +ACM/GM-CSF
33
BMC +ACM +GM-CSF
BMC +Flt3L +ACM +GM-CSF
Events
Fluorescence
Primary Microglia
BMC +ACM/GM-CSF
BMC +Flt3L/ ACM/GM-CSF
BMC +Flt3L
BMC 17 days
BMC 0 days
Phagocytosis [percent positive cells]
Oxidative Burst [Fluorescence signal to noise ratio]


100 µm
A: Primary microglia
20 µm
B: Primary microglia
C: BMC day 17
D: BMC day 11
E: BMC +Flt3L day 11
F: BMC +ACM/GM-CSF day 17
G: BMC +Flt3L day 17
H: BMC+Flt3L+ACM/GM-CSF day 17


A: day 3 BMC+ACM/GM-CSF
B: day 6 BMC +ACM/GM-CSF
C: day 10 BMC +ACM/GM-CSF
160 µm
D: day 1 BMC+ACM/GM-CSF
160 µm
E: day 2 BMC+ACM/GM-CSF
160 µm
F: day 3 BMC+ACM/GM-CSF


A: BMC
B: BMC+Flt3L
160 µm
160 µm
C: BMC+Flt3L+ACM/GM-CSF
D: BMC+ACM/GM-SCF
160 µm
160 µm

4. Paper 2: “Microglia differentiation using a culture system for the expansion of mice non-adherent bone marrow stem cells.”

Microglia differentiation using a culture system for the expansion of mice non-adherent bone marrow stem cells

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Introduction: Studying primary adult microglia is hampered because of the difficult isolation procedure and the low cell yield. We therefore established a differentiation protocol using a culture system developed for the expansion of non-adherent bone marrow cells. **Methods:** Non-adherent bone marrow derived stem cells (NA-BMC) are derived by selective adhesion ('preplating') and are non adhesive adult stem cells. We investigated the changes in bone marrow cell populations by this repeated selective adhesion and compared the potential of the derived cells to differentiate towards microglia. Cells were differentiated with astrocyte conditioned medium (ACM) and granulocyte-monocyte colony stimulating factor (GM-CSF). **Results:** NA-BMC cultures show a steep raise in the fraction of stem cells during the cultivation time and the differentiation potential is of the same quality as established protocols. Around 70% of the cells are microglia defined as being positive for CD11b/CD45 and show phagocytosis activity and oxidative bursts. **Conclusion:** The non-adherent cell system has the advantage that is produces stem cell progenitors during expansion and provides good microglial differentiation.

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Key words: non-adherent stem cells, bone marrow, microglia, differentiation

Introduction

Microglia are the immune-cells of the brain. They react to inflammatory signals, seek out and phagocytize debris and promote repair and regeneration by excreting growth factors [1]. Microglia might, however, promote neurodegenerative diseases like Alzheimer or multiple sclerosis by dysregulation and overreaction to chronic inflammatory signals. A suspected loss of their ability to function as debris clearing cells with age or an insufficient renewal of their population in age was described [1]. The role microglia play in these diseases and the possible changes the microglia population undergoes with age are the key to understand and combat the causes of neurodegeneration.

Studies of microglia *in vitro* use mostly primary microglia from mouse or rat embryos. Human microglia are difficult to obtain and they are often derived from post-mortem donors, posing some extra difficulties concerning cell viability. To study the role of microglia in neurodegeneration it is however necessary to work with adult material as the onset of the disease is age-dependent. The alternative is to use blood or bone marrow derived monocytes to derive microglia [2]. The *in vitro* generation of microglia may chart the way towards new therapeutic strategies using adult stem cells or to study the function of microglia generated from individuals of all ages and from diseased background to better understand their role in neurodegeneration.

Non-adherent bone marrow cells (NA-BMCs) harbor cells of the hematopoietic lineage [3]. NA-BMCs are known to rescue lethally irradiated mice [3, 4]. Their potential to give rise to microglia could be of use in cell-based therapies of the central nervous system (CNS) [1]. Non-adherent mesenchymal stem cells (MSC) are present in NA-BMC cultures as well [3]. They give rise to fibroblastic, osteoblastic, chondrocytic and adipocytic lineages. They have been found to colonize various tissues like bone marrow, spleen, intestine, kidney and liver.

NA-BMCs might correspond to a naturally circulating population of cells [3], which carries progenitors of several somatic cell types. In line with this, cells residing in the blood have been differentiated to various cell types [5] and it is known that peripheral blood monocytes can be differentiated to microglia *in vitro* [6]. Also, fibroblast-like cells are mobilized from the bone marrow by various effects like cytokines, hypoxia and skin damage [7-10].

MSC are known to regulate microglia and other immune cells [11-14]. Bone marrow derived mesenchymal stem cells transplanted into the brain result in a reduction of amyloid-ß plaques [15] - possibly by activation of resident microglia to an amoeboid and phagocytic state. Transplanted MSC have positive effects in neurodegeneration by regulation of the microenvironment and by cell fusion [16, 17]. MSC regulate activation of microglia in co-culture [18, 19]. These findings suggest that inclusion of MSC in a microglia cell population might even be beneficial.

To solve the problem of microglia availability and viability we explore here the improved generation of microglia cells from adult stem cells. We designed two new protocols to differentiate microglia from so called non-adherent bone marrow cells (NA-BMCs) (Fig. 1), which can be expanded efficiently in vitro in suspension cultures without loss of stem cell properties. They correspond to a classical method for macrophage differentiation (Protocol 2) and a culture system originally developed for expansion of stem cells (Protocol 1) (Fig. 1). The different stages and gradual changes during this expansion protocol are poorly investigated. The composition of these cell cultures over time, the changes in colony forming units (CFU-f) and the capacity to differentiate to microglia have not been characterized in the past. Special focus was on the functional characterization of the microglia derived by this protocol.

Materials and Methods

Animals

Animals used for the experiments were C57BL/6 from the MEZ Leipzig and Charles River (Sulzfeld, Germany). They were handled in accordance to local animal ethics regulations.

Bone marrow isolation and culture of NA-BMC

Femurae and tibiae of 2-3 month old C57BL/6 mice were isolated, opened and centrifuged to obtain bone marrow. 10^7 bone marrow cells were cultivated for 24h in a 60mm petri dish and in 10ml Dulbecco's modified eagle medium (low glucose) (DMEM, Hyclone Laboratories Inc.), supplemented with 10% fetal calf serum (FCS) (Invitrogen), 10^{-8} M dexamethasone and 100 units/ml Penicillin/Streptomycin (Invitrogen). After 24h the non-adherent cells were flushed off and transferred to a new dish (protocol 2; Fig. 1). This 24h adhesion period was repeated 4 times to derive NA-BMC cells (protocol 1; Fig. 1).

CFU-f

The non-adherent cells of day 1 (classical replating protocol to derive macrophages, protocol 2) and NA-BMCs from day 4 (protocol 1) were resuspended in 5ml osteogenic medium (DMEM, 10% FCS, 10^{-8} M dexamethasone, 50µg/ml ascorbic acid) in a 60mm dish. Every 3 days, the medium was changed. After 10 days, the cells were fixed with cold ethanol and alkaline phosphatase (ALP), calcium (Alizarin red), collagen (Sirius red) and methylene blue (total colonies) staining performed. The colony numbers were determined using the program ImageJ.

ALP staining

The cells in 60mm petri dishes were fixed with cold ethanol for 15min. They were washed with tap water. Tris (200mM, pH 8.5) was mixed with naphthol phosphate ASBI (50µg/ml) and fast red (1mg/ml) (Fast red was always mixed fresh). 5ml of the mixture was added to petri dishes. The dishes were shaken for 2h at room temperature. Afterwards they were washed with tap water and allowed to dry. Photographs of the dishes were taken and colony numbers determined with ImageJ.

Alizarin red staining (Calcium)

Cells were fixed for 15min with ice-cold ethanol, afterwards washed with tap water. 5ml of a solution of 1mg/ml alizarin red in distilled water, pH 5.5 were added. The petri dishes were shaken for 2h, afterwards washed with tap water and allowed to dry. Images were analyzed for calcium amount using ImageJ.

Sirius red staining (Collagen)

Cells were fixed in ice-cold ethanol for 15min and washed with tap water. 1mg/ml sirius red was solved in picric acid. 5ml of the mixture was added to the cells and the petri dishes shaken for 18h at room temperature. Then the cells were washed with tap water till red color was completely eluted. The dishes were photographed and analyzed for the amount of collagen (ImageJ).

Methylene blue staining (total colony numbers)

Cells were fixed in ice-cold ethanol for 15min. They were washed in tap water. 1mg/ml methylene blue was solved in 10mM borate buffer, pH 8.8. 5ml of the mixture was added to the petri dishes and shaken for 30min. The dishes were washed with tap water until all dye was eluted, photographed and analyzed using ImageJ.

Astrocyte conditioned medium

Astrocyte conditioned medium was produced by incubating medium (DMEM/10% FCS) 24h with primary astrocyte cultures produced as described by Sievers [2].

Differentiation towards microglia-like cells

On day 1 (protocol 2) and day 4 (protocol 1), the non-adherent cells were flushed off and transferred to a new dish. Afterwards cells were differentiated for 6 days in 10ml

DMEM/10% FCS, 50% ACM and 20ng/ml granulocyte-monocyte colony stimulating factor (GM-CSF). Controls were cultured only in DMEM/10% FCS.

Flow cytometry

Cell were stained for F4/80 (AF488 labelled, eBioscience) (1:250), CD11b/CD45 (AF488 and PE labelled eBioscience) (1:250 and 1:100) and CD34 (PE labelled, Caltag Laboratories) (1:100), CD45 R (RPE labelled, Southern Biotech) (1:100). Cells were trypsinized, centrifuged (300g for 5min) and fixed (4% paraformaldehyd). The fixed cells were washed with PBS, incubated for 2h at 4 °C with primary antibody, washed and analysed in a Beckmann Coulter FC 500 Flow cytometer.

Phagocytosis

$3*10^5$ cells were activated with 0.1µM phorbol myristic acid for 15min. Then they were incubated in 50µl DMEM/10% FCS together with 50µl 1:10 diluted opsonised beads ($2.25*10^7$ beads) (Sigma) for 48h at 37°C, 5% CO_2. Cells were trypsinized and resuspended in DPBS (Invitrogen) and fluorescence was measured in a Beckmann Coulter FC 500.

Oxidative burst

$3*10^5$ cells were activated for 15min with 0.1µM PMA and controls without PMA. Activated and control cells were incubated with 50µM DHR123 for additional 15min. Afterwards the cells were fixed with 4% PFA and fluorescence was measured in a Beckmann Coulter FC 500. Oxidative burst was defined as signal to noise ratio (The ratio of fluorescence of activated to control cells).

Living brain slice cultures

The brain from 2-3 month old C57BL/6 mice were transferred to cold Hank's buffered salt solution (HBSS)/10% FCS (both Invitrogen). A VT 1000 S vibratome (Leica) was used to cut the brain in 350µm slices. The slices were culture on a membrane (Millicell CM 0.4µm, Millipore) at the liquid air interface of medium consisting of 50% DMEM/high glucose (HyClone Laboratories Inc.), 25% horse serum (Invitrogen), 25% HBSS (Invitrogen), 1µg/ml insulin (Invitrogen), 100units/ml penicillin and 100µg/ml streptomycin (Invitrogen). The brain slices were cultured for 9 days and the viability of the brain slices were assessed using DAPI/propidium iodide staining. For analysis the slice was scan using a confocal microscope (TCS SP2, Leica Microsystems).

Invasion of living brain tissue

On day 9 of brain slice culture, differentiated cells were treated with DIO (Invitrogen) for 20min, washed and seeded on the top of the brain slices. A plastic ring was used to keep the cells from flowing off the slices. Cells and brain tissue were co-cultivated for additional 10 days [20]. After 10 days migration of cells into brain tissue was measured by scanning the slices with a confocal microscope (TCS SP2, Leica Microsystems) to a depth of 160µm.

Statistical Analysis

Data is presented as means ± SE. SigmaPlot 10.0/SigmaStat 3.5 software (SYSTAT, Erkrath, Germany) was used to perform statistical analysis. For comparison of different groups ANOVA was used.

Results

Cell populations during selective adhesion and differentiation

The numbers of non-adherent cells in culture fell swiftly from day 0 to day 4 of selective adhesion (Fig. 1 B). From whole bone marrow, after 1 day of selective adhesion $3.5*10^6$ non-adherent cells remained. After 4 days of selective adhesion cell yield was $1.7*10^6$ non-adherent cells.

The NA-BMC cultures were analysed at different days using flow cytometry (Fig. 2 A). The prominent cell populations – of immature/nucleated red blood cells, lymphocytes, monocytes and granulocytes – were defined according to their forward and side scattering (Fig. 2 A) as done by other groups [21]. The fraction of these populations changed during selective adhesion (Fig. 2 B). The fraction of lymphocytes decreased significantly from 35.6% to 10.7% during the 4 transfers of non-adherent cells ($P<0.05$). The population of immature and nucleated red blood cells (NRBC) increased from 28.2% to 50.8% ($P=0.13$). Monocyte and granulocyte populations did not change during the cultivation (Fig. 2 B). The cells derived using protocol 2 still had a prominent lymphocyte population while the cells from protocol 1 lacked lymphocytes (Fig. 2 A and B). The supernatant of each day was used for colony forming unit (CFU-f) assays. The frequency of methylene blue positive colonies doubled ($P<0.05$), alkaline phosphatase (ALP) and calcium positive colonies tripled ($P<0.01$), while collagen positive colonies did not change significantly (Fig. 2 C).

Marker expression levels

As this technique was not used before for the derivation of microglia, we wanted to describe the cultures in some detail and the changes occurring over time. During the selective adhesion period, using protocol 1, cell granularity doubled and the mean cell size decreased 3 fold (Fig. 3). We were especially interested in the frequency of cells expressing macrophages/microglia markers and hematopoietic progenitor markers. The median of CD34 expression tripled significantly ($P<0.05$), as well as 4-fold CD45 R expression ($P<0.05$) and doubled F4/80 expression ($P<0.05$). No change of CD45 expression was observed. CD11b expression decreased, significantly, 3-fold ($P<0.05$) during the 4 days of selective adhesion (Fig. 3).

Microglia differentiation

The selection of non-adherent cells by adhesion was combined with differentiation media. Our new cell culture protocol (protocol 1) was compared with the classical approach (protocol 2).

It is difficult to differentiate between macrophages and activated microglia. We used a combination of non-exclusive markers and typical morphology (ramification) as specific signs of microglia (resting macrophages) as suggested by [22]. In addition we used the combination of CD11b/CD45 expression as employed by other groups [22, 23] and the macrophage marker F4/80.

The cells derived using protocol 1 and 2 were differentiated to microglia with astrocyte conditioned medium (ACM) and granulocyte-monocyte colony stimulating factor (GM-CSF) supplementation for 6 days. The cells differentiated using protocol 1 were as efficient producing microglia-like cells as protocol 2 – judged by the marker combinations suggested to be specific for microglia (Fig. 4 and Fig. 5).

Functional tests

Microglia derived using protocol 2 showed a significantly higher phagocytosis rate (1/4 higher) than the cells from protocol 1 (Fig. 6 A). Supplementation of ACM and GM-CSF lead to a significant, 4 fold increase in the phagocytic ability of the differentiated cells in both protocols. It is interesting to notice that cells of both ACM/GM-CSF supplemented and unsupplemented cultures show almost the same marker expression (Fig. 4 BCD, Fig. 5 C) but differ in phagocytosis (Fig 6 A).

Microglia from cultures of protocol 1 showed the same capacity for an oxidative burst as microglia derived from protocol 2 cultures (Fig. 6 B). Cytokine supplementation significantly increased oxidative burst in cells derived using protocol 2 - that was present both in supplemented and unsupplemented microglia from protocol 1.

Morphology

Cells derived from protocol 1 show many small and non-adherent cells and all differentiated cell populations show mixed morphologies as is typical for microglia cultures (Fig. 6 C-F). Extensive ramification was, however, not visible.

Organotypical brain slices

The cells differentiated with the new protocol 1 invaded living brain tissue to a depth of up to 80 µm (Fig. 6 J). Most of the cytokine differentiated cells and the un-supplemented cells migrated into the surface of the slices to a depth of 30 µm.

Cells differentiated from classical derived macrophage showed amoeboid morphology (Fig. 6 I, J). Cells differentiated from NA-BMCs showed round morphologies (Fig. 6 G, H). In our qualitative migration experiments cells treated with ACM/GM-CSF or cells derived from early NA-BMCs migrated deepest (Fig. 6 J).

Discussion

Microglia derived from adult material are difficult to obtain. Most studies have been performed on microglia from neonatal sources and cell yields from adult sources are low. In settings where cells would be used clinically, autologous sources are preferable. Bone marrow cells are routinely used for transplantation and contain different stem cell types. We have established here a protocol for the derivation of functional microglia using adult bone marrow providing a source for cell therapy or drug development. It is assumed that in the healthy brain microglia are replenished locally but that under pathological conditions and inflammation bone marrow derived cells can invade the brain and differentiate to microglia [24].

We used a stem cell cultivation method which was originally developed to expand the undifferentiated stem cell population [3, 25] and tested their use as source for microglia. Repeated selective adhesion, as employed to derive non-adherent bone marrow cells (NA-BMCs), result in a rising capacity of NA-BMCs to form CFU-f. This as well as the high CD34 expression shows that the frequency of stem cells or progenitors increases during time in these cultures. The lymphocyte population in the new suspension cultures (protocol 1) gets diminished by the repeated adhesion and the fraction of immature cells increases. This corresponds to results from other groups showing in rats the increase of CFU-f initiating cells during the repeated re-plating steps [3]. The results for the monocyte lineage were mixed and we can not conclude if during the initial culture phase the numbers of progenitors for microglia are diminished or not.

Microglia differentiation: No single marker distinguishes microglia from macrophages. Some publications suggest that CD45 and CD11b together might distinguish between microglia and macrophages [26]. CD45 is a marker for the hematopoietic cells and F4/80 for microglia and macrophages [27]. Our new protocol using non-adherent bone marrow cells showed the same level of differentiation towards microglial cells expressing $CD11b^{+}/CD45^{low}$ and $F4/80^{+}$, which, together with morphology (ramification), define the microglia population as described by previous studies [22, 23]. Functional microglia derived using the new protocol 1 showed slightly less phagocytosis activity than those of protocol 2, but the burst activity was of the same level.

The lower phagocytosis of cells from protocol 1 might be due to less differentiated and more primitive cells in the supernatant of day 4. It might also be an effect of the longer time in culture before differentiation. That phagocytosis is low in absence of cytokine supplementation is known [28]. Oxidative burst of the differentiated cells is not significantly higher than that of whole bone marrow. Although there are subpopulations in whole bone marrow that do show strong oxidative burst this might point to an immature microglia cell type [29].

Morphologies were mixed and only few cells showed clearly branches typical for primary resting microglia as one would expect when cells were incubated with astrocyte conditioned medium. The amoeboid and round cell morphology in the co-culture with brain slices indicates an active state in all tested protocols. This might be induced by the apoptotic cells in the slice. It is known that microglia of the brain slices migrate to the surface and phagocytize debris there [30].

Conclusion: Repeated selective adhesion enriches for stem cells and provides great numbers of microglia, which are functionally active. This protocol provides functional adult microglia which can be used for drug development or cell therapies.

Competing interests

No competing interests.

Authors' contributions

AH carried out all experiments and wrote the manuscript. AS designed & coordinated the study and contributed to writing the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1: (A) Overview showing the two differentiation methods. Representative forward scatter (FSC) and side scatter (SSC) plots of NA-BMC are shown. **Day 0 – day 4:** Phase of selective adhesion. **Day 4 - day 10 and day 1 - day 7:** Differentiation phase. **(B)** Yield of non-adherent cells per whole bone marrow on day 1 – day 4. Repeated selective adhesion results in a falling number of cells.

Fig. 2: (A) Scatter plots of NA-BMC during the selective adhesion phase. The indicated regions define populations of immature and nucleated red blood cells, lymphocytes, monocytes and granulocytes. P is the probability of the null hypothesis of the linear regression, i. e. that the percentage fraction does not change over time. **(B)** Changes in the percentage fraction of the cell populations is shown. Lymphocyte fraction is diminished by selective adhesion. **(C)** CFU-f grown from NA-BMCs of day 1 to day 4. Methylene blue staining was used to determine total colonies. Alizarin red (Calcium), Sirius Red (Collagen) and alkaline phosphatase (ALP) staining were used to detect the capacity of NA-BMC for osteogenic differentiation.

Figure 3: Median of NA-BMC antigen expression during day 0 – day 4 of selective adhesion. Both markers of differentiated (F4/80) and undifferentiated hematopoietic cells (CD34) rise. Cell size gets smaller, larger cells seem to become adherent. The

regression tests for a statistically significant change over time. P is the probability of the null hypothesis – that marker expression does not change over time.

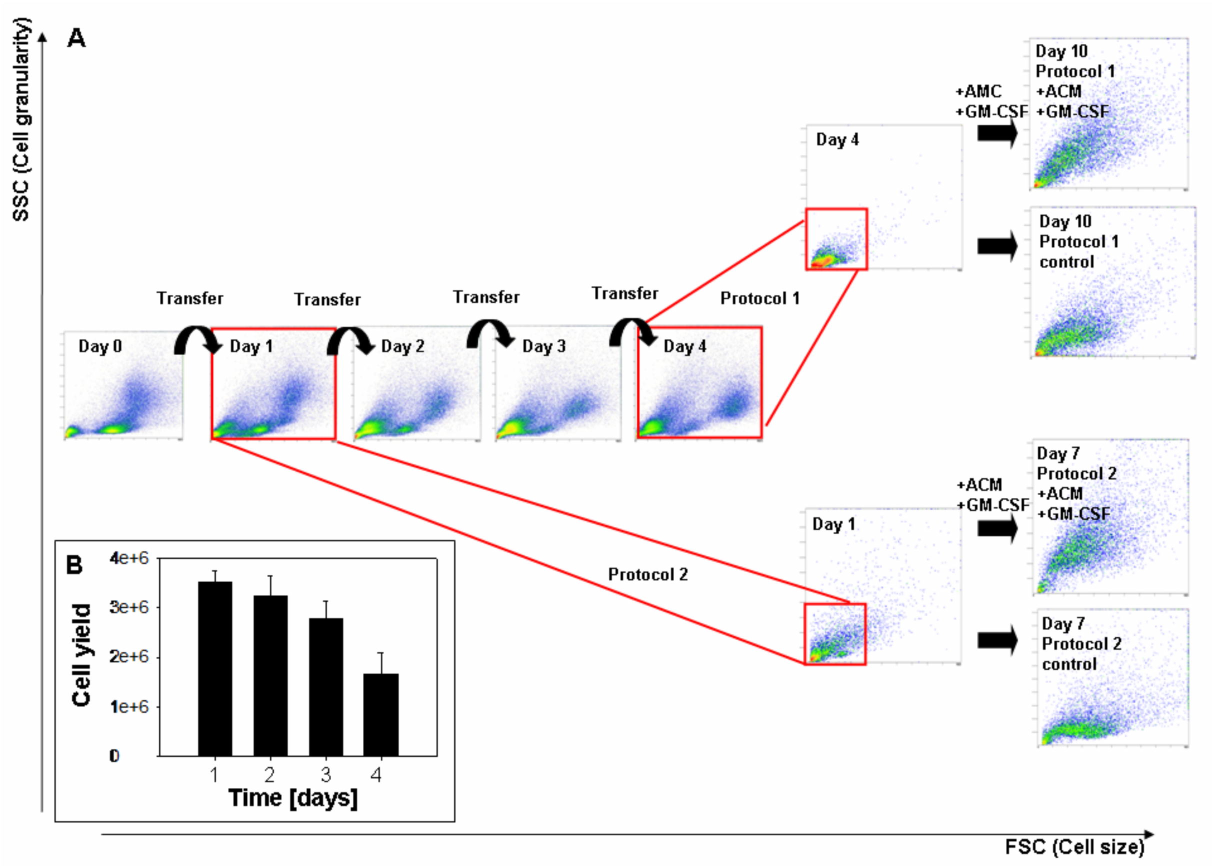
Fig. 4: (A) Representative scatter plots of differentiated cells. A microglia gate was set, defined by the primary microglia population, to assess differentiation. **(B)** Percent of CD11b/CD45low (microglia gate) among the differentiated cells. Yields of cells showing the microglia markers are not different between the two protocols. A small subpopulation in fresh bone marrow (20%) shows microglia markers from the beginning. **(C)** and **(D)** Percent of $CD11b^{+}$ and $CD45^{+}$ cells among the differentiated cells. CD45 is already high in fresh bone marrow. P1: Protocol 1. P2: Protocol 2. *** = $P < 0.001$, ** = $P < 0.005$, * = $P < 0.01$

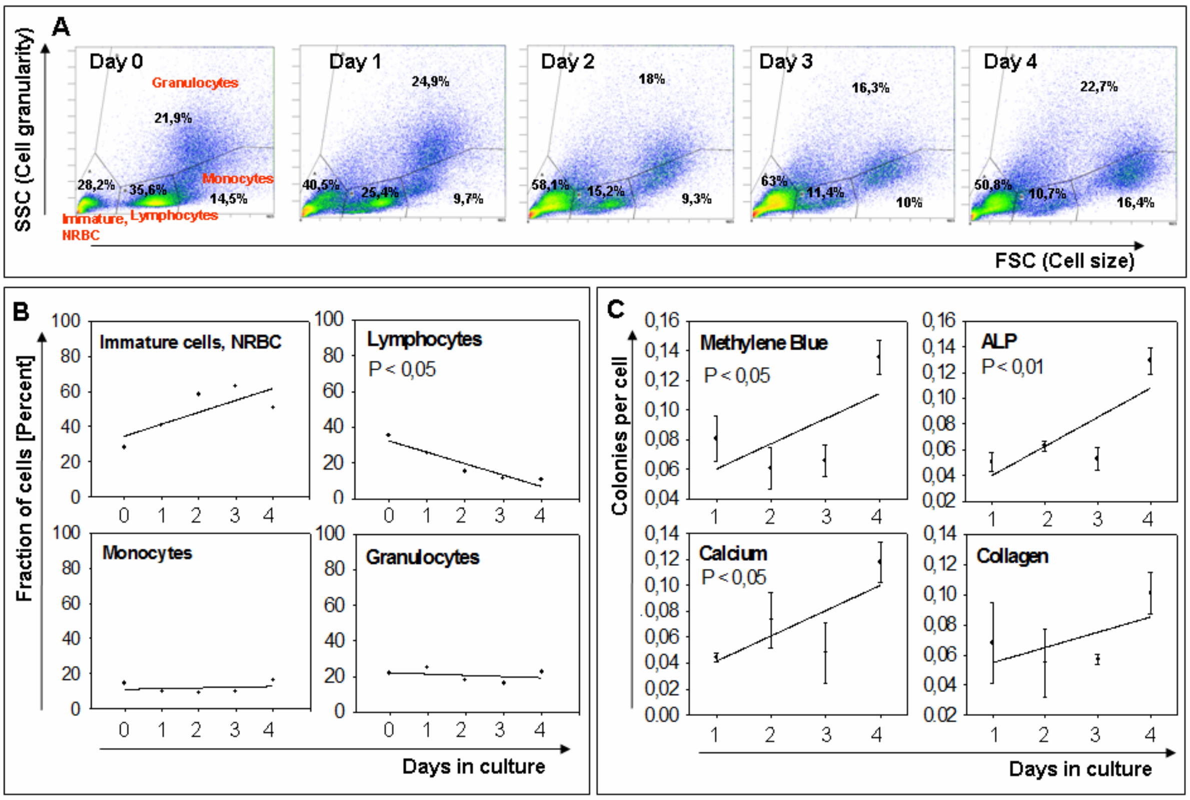
Fig. 5: (A) Representative histogram plots of F4/80 expression. Gray line is the isotype control. **(B)** Percent of $F4/80^{+}$ cells among the differentiated cells. Protocol 2 shows higher F4/80 expression than protocol 1 although, during selective adhesion, F4/80 rose steadily. P1: Protocol 1. P2: Protocol 2. *** = $P < 0.001$, ** = $P < 0.005$, * = $P < 0.01$

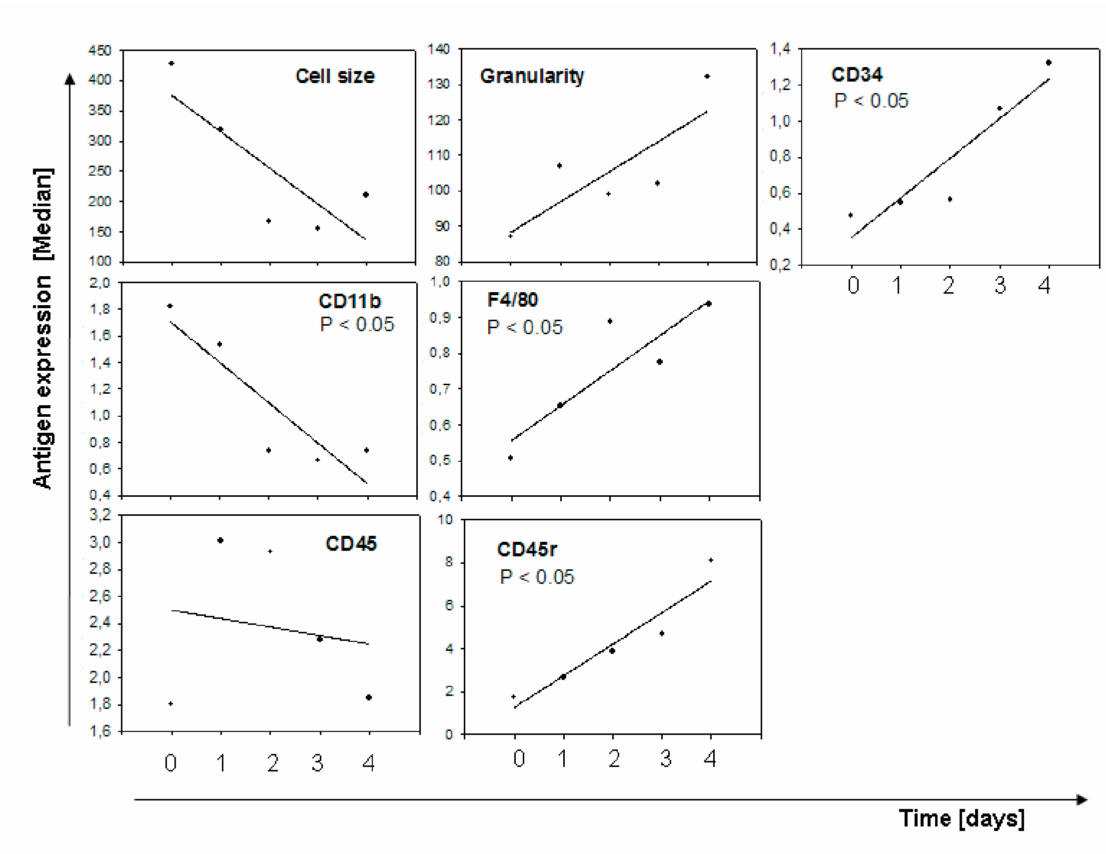
Fig. 6: (A) Phagocytic activity of differentiated cells. Cells of the classical pre-plating protocol 2 show significantly higher phagocytosis of fluorescent beads. Cells un-supplemented with ACM and GM-CSF show low phagocytosis. ($n = 3$). **(B)** Oxidative burst of the differentiated cells is not different between protocol 1 and protocol 2. P1: Protocol 1. P2: Protocol 2. ($n = 3$).

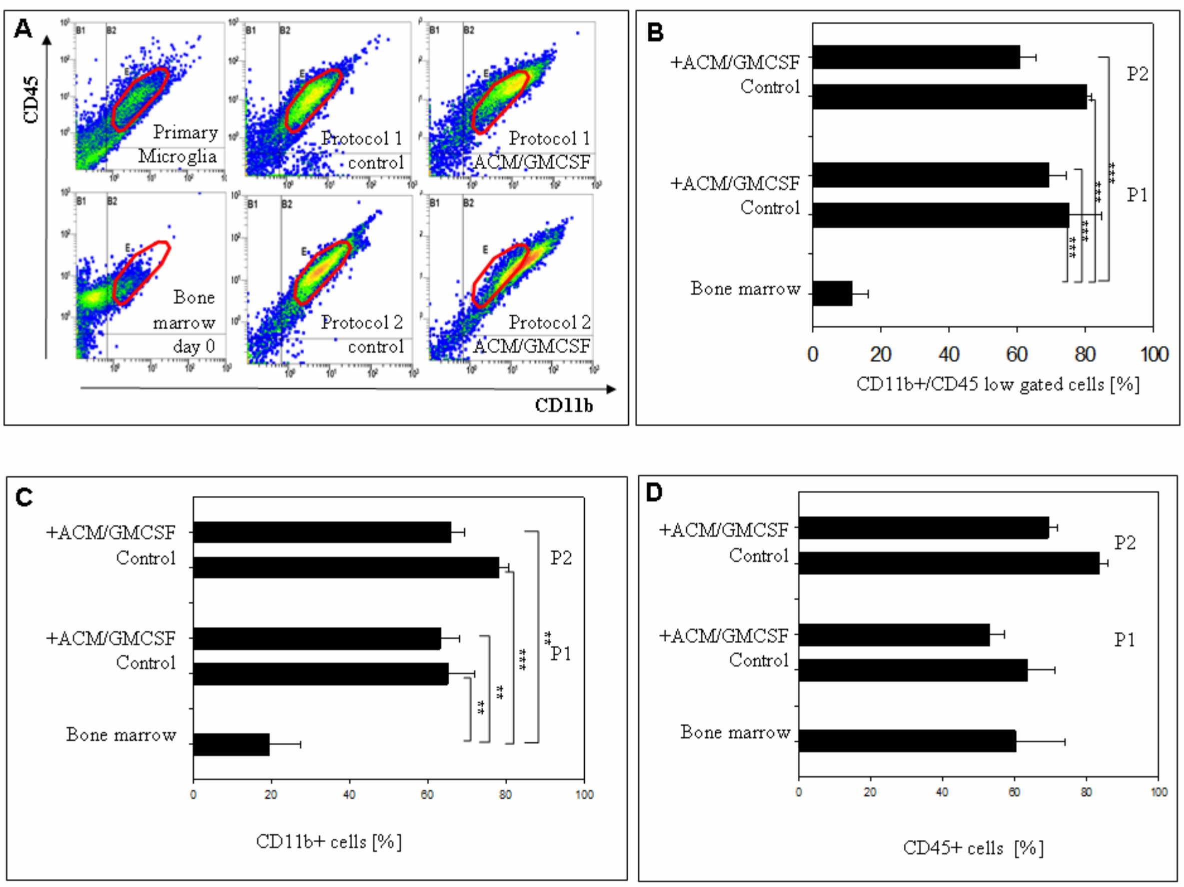
(C-F) Differentiated cells of protocol 1 are smaller and contain many non-adherent cells. Morphologies and resulting cell types are diverse. Images were taken with a Leica DMIL at 200x magnification.

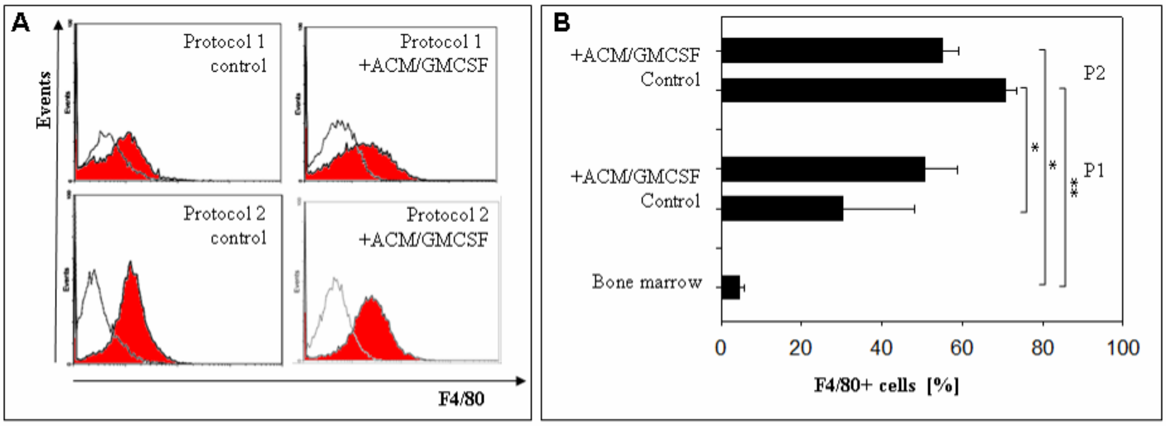
(G-J) Most differentiated cells survive in co-culture and remain largely amoeboid and round, typical for activated states. The cells were co-cultured with living brain slices. Top down and side view pictures. Differentiated cells were labeled green (DIO) and transferred onto 9 day old living brain slices. Dead cells were labeled red (propidium iodide). After 10 days of co-culture a Leica Microsystems SP2 confocal microscope was used to scan the brain slices to a depth of 160 µm (Magnification 100x). **(H, J)** Cells of the cytokine supplemented cultures invade surface up to a depth of 60 µm. *** = $P < 0.001$, ** = $P < 0.005$, * = $P < 0.01$

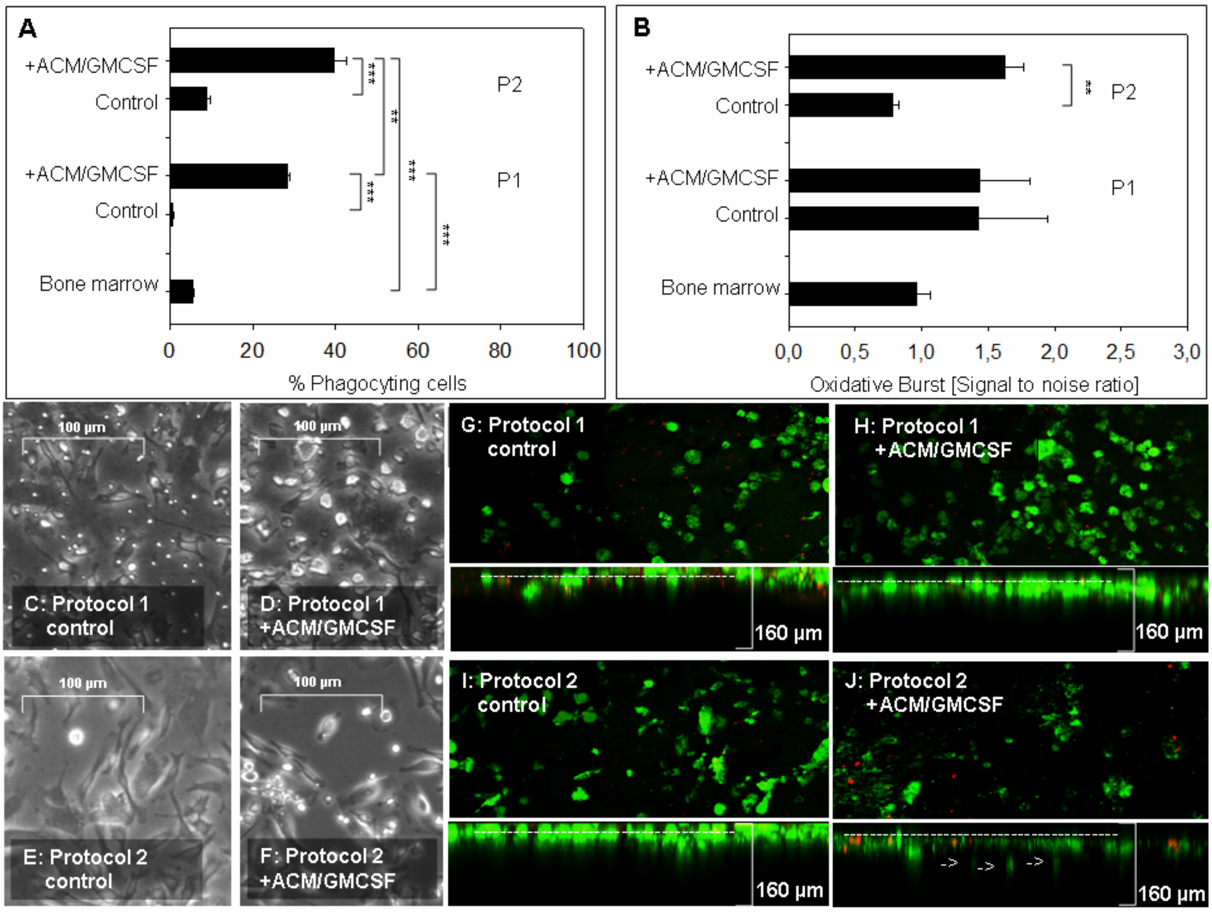

A
SSC (Cell granularity)
Day 0
Transfer
Day 1
Transfer
Day 2
Transfer
Day 3
Transfer
Day 4
Protocol 1
Day 4
+AMC
+GM-CSF
Day 10
Protocol 1
+ACM
+GM-CSF
Day 10
Protocol 1
control
Protocol 2
Day 1
+ACM
+GM-CSF
Day 7
Protocol 2
+ACM
+GM-CSF
Day 7
Protocol 2
control
B
Cell yield
4e+6
3e+6
2e+6
1e+6
0
1 2 3 4
Time [days]
FSC (Cell size)


A
SSC (Cell granularity)
Day 0
Granulocytes
21,9%
28,2%
35,6%
Monocytes
14,5%
Immature, NRBC
Lymphocytes
Day 1
24,9%
40,5%
25,4%
9,7%
Day 2
18%
58,1%
15,2%
9,3%
Day 3
16,3%
63%
11,4%
10%
Day 4
22,7%
50,8%
10,7%
16,4%
FSC (Cell size)
B
Fraction of cells [Percent]
Immature cells, NRBC
Lymphocytes
P < 0,05
Monocytes
Granulocytes
Days in culture
C
Colonies per cell
Methylene Blue
P < 0,05
ALP
P < 0,01
Calcium
P < 0,05
Collagen
Days in culture


Antigen expression [Median]
Cell size
Granularity
CD34
P < 0.05
CD11b
P < 0.05
F4/80
P < 0.05
CD45
CD45r
P < 0.05
Time [days]


A
CD45
Primary Microglia
Protocol 1 control
Protocol 1 ACM/GMCSF
Bone marrow day 0
Protocol 2 control
Protocol 2 ACM/GMCSF
CD11b
B
+ACM/GMCSF
Control
P2
+ACM/GMCSF
Control
P1
Bone marrow
CD11b+/CD45 low gated cells [%]
C
+ACM/GMCSF
Control
P2
+ACM/GMCSF
Control
P1
Bone marrow
CD11b+ cells [%]
D
+ACM/GMCSF
Control
P2
+ACM/GMCSF
Control
P1
Bone marrow
CD45+ cells [%]


A
Events
Protocol 1
control
Protocol 1
+ACM/GMCSF
Protocol 2
control
Protocol 2
+ACM/GMCSF
F4/80
B
+ACM/GMCSF
Control
+ACM/GMCSF
Control
Bone marrow
P2
P1
*
*
**
0
20
40
60
80
100
F4/80+ cells [%]


A
+ACM/GMCSF
Control
+ACM/GMCSF
Control
Bone marrow
P2
P1
***
**
***
***
***
0
20
40
60
80
100
% Phagocyting cells
B
+ACM/GMCSF
Control
+ACM/GMCSF
Control
Bone marrow
**
P2
P1
0,0
0,5
1,0
1,5
2,0
2,5
3,0
Oxidative Burst [Signal to noise ratio]
100 µm
C: Protocol 1
control
100 µm
D: Protocol 1
+ACM/GMCSF
G: Protocol 1
control
160 µm
H: Protocol 1
+ACM/GMCSF
160 µm
100 µm
E: Protocol 2
control
100 µm
F: Protocol 2
+ACM/GMCSF
I: Protocol 2
control
160 µm
J: Protocol 2
+ACM/GMCSF
->
->
->
160 µm

5. Paper 3: “To Migrate or not to Migrate – Microglia and Cell Migration to and from the Brain.”

To Migrate or not to Migrate – Microglia and Cell Migration to and from the Brain

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Abstract

Microglia are the resident phagocytes of the brain. They play an important role in several age related diseases. A loss of function of aged microglia and replicative senescence might contribute to diseases like Alzheimer or Multiple Sclerosis. However, a cell replacement therapy is difficult because cell migration in and out of the healthy, non-diseased brain is absent or low. This is a considerable challenge for the application of cells. Therefore, we provide here an overview of parameters and conditions that influence migration to and from the mouse brain.

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Key words:

Microglia, Migration, Brain

Introduction

Microglia: Microglia comprise about 10% of the cell population of the brain [1]. They perform phagocytosis, are cytotoxic, present antigens and promote repair after injury [2].

Under healthy conditions, microglia are quiescent. Microglia functions like phagocytosis, immune response and migration are down-regulated. The cells are ramified and display long and branched processes [3, 4].

Microglia are often compared to monocytes or viewed as immature macrophages because of their differentiation properties [5]. This definition is somewhat fluid because of the lack of distinct properties or markers – apart from morphology – that set these cell populations apart.

Microglia in Disease: In response to inflammation, microglia become 'activated'. Their morphology changes to an amoeboid shape in mice. Proliferation is up-regulated by inflammation [6-8]. Microglia migrate, over the course of 1-2 days, towards injury and extracellular debris [9].

The general quiescence and graded up-regulation of microglia activity might exist to minimize damage to neighboring neural tissue if an immune response is initiated [10]. If microglia are chronically activated, they have been observed to act cytotoxic and damage cells in the vicinity [11].

Microglia in Age: In brains of older humans, deterioration of microglia is observable [12]. In the aging human brain, dystrophic microglia are present which show de-ramification, atrophy, fragmentation and swelling [12]. *In vitro* cultured microglia, if exposed to amyloid protein, are degenerating faster [13]. This has also been reported by others, and an overload of the intracellular mechanisms to break down digested proteins (proteasome) might be responsible [14-16].

Microglia in Therapy: Migration to and from the healthy, young brain is absent or low [17]. Such cell migration is influenced by a wide range of parameters and conditions like injury and cell death in the brain, resulting migration signals, the permeabilization of the blood brain barrier due to various conditions like irradiation, toxins and age. There are, furthermore, indications that stem or progenitor cells in the bone marrow are necessary for migration into the brain [18]. In a therapy, the treatment of cells prior to application, application routes but also cell dose, survival, *in vivo* proliferation and many other factors play a role (Table 1).

Migration during Development

Colonization of the Brain: The central nervous system (CNS) is populated by microglia progenitors on (embryonic) days 10-19 in rodents [19]. In man, this colonization takes place between pre-natal months 3-5. Microglia appear within CNS before vascularization [20] and before monocytes are produced in hemopoietic tissues [5, 20]. This seems to occur partially in response to cell death and related signals [20].

The natural routes on which microglia precursors might migrate into and colonize the brain are the ventricular layer, the pial surface of the meninges, and here especially the areas of the optical tectum and cerebellum, and the endothelial wall of the later established vasculature (Fig. 1) [19, 20]. Cells adhere to the endothelial walls of the bloodvessels and ‘squeeze through’ the endothel much like macrophages and monocytes do in other tissues (Ibid.). For microglia precursors there is evidence that adhesion is mediated by LFA-1/ICAM-1 (lymphocyte function-associated antigen-1/intercellular adhesion molecule-1) [21]. At the time microglia precursors invade the developing brain, ICAM-1 expression in the endothel and LFA-1 positive cells are present in the vessel. Furthermore, microglia seem to cross the ventricular layer during early development [22]. Similar observations exist for prominent points of the pial border and microglia in the optic tectum and cerebellum might derive from such sources [20].

In the adult organism, in principle, the same routes of migration to and from the brain would be possible – however, the endothelial wall becomes much less permeable in the mature brain (blood-brain-barrier).

Migration under Healthy Conditions

Migration in the Brain: Under healthy conditions, microglia are ramified. It has long been thought that in this state microglia are virtually non-motile. However, *in vivo* motion picture observations have shown that microglia are in fact continuously forming protrusions that are extending and withdrawing [3]. This is similar to extensions in macrophages, which act to draw debris to the cell body for phagocytosis. The long and branched processes of ramified microglia might monitor the local neighborhood in this way.

Microglia are activated through distinct pathways and can respond both anti- and proinflammatory [15]. If microglia encounter cell debris, receptors recognizing phosphatidylserine in apoptotic membranes drive their response (Ibid.). Other groups emphasize the importance of oxidized proteins and lipids in apoptotic cells and their recognition by vitronectin receptors, scavenger receptor B1 (CD36) and CD14 [23]. Many receptors seem to be involved in these mechanisms and seem to be highly redundant (Ibid.). They might also help in ‘tethering’ the apoptotic cell while only certain receptors signal via associated proteins [24].

The loss of function of certain receptors like the triggering receptor (TREM2) or DNAX-activating protein 12kDa (DAP12) has been observed to lead to an inflammatory neurodegenerative disease later in life [25]. Tim4 (T-cell immunoglobulin- and mucin-domain-containing molecule-4) recognizes phosphatidylserine [26]. Purine receptors active in phagocytosis of microglia like P2Y6 recognize UDP from injured neurons [27].

The removal of such ‘normal’ debris takes place without full activation or strong movement of the cell body. Here, microglia perform a ‘silent’ phagocytosis [15] and act anti-inflammatory. They show anti-inflammatory cytokines like tumor growth factor beta (TGF-beta) and interleukin 10 (IL-10) [24].

The pro-inflammatory pathway is discussed in the chapter covering diseased conditions.

The Blood Brain Barrier: One of the routes to enter the brain is migration through the bloodstream. Because of the differing specialization of organs, blood vessels are adapted to that function. For example organs, which have filtering function (liver), or provide red blood cells (bone marrow), display porous or even open vessels. The CNS however is shielded from the bloodstream by a tight endothelial barrier, the so called blood brain barrier (BBB) [28, 29]. Some parts of the brain, the circumventricular organs, are not covered by the blood brain barrier [30]. Examples are the pituitary gland, that secretes hormones like oxytoxin or vasopressin endocrinal, or the area postrema .

This barrier serves as a protection from drugs and harmful substances, infections and overreaction of the immune system. It only becomes more permeable under pathological conditions. Therefore, it has been discussed controversially whether, later in life, a migration of cells through this barrier takes place.

Migration across the BBB: In unlesioned hemipheres of the brain, migration of bone marrow cells is restricted to sites of macrophage turnover like meninges and the Virchow-Robin spaces (Fig. 2) [31]. Without irradiation, cell migration to the brain is absent or low [18]. Massberg et al. observed that parabiotic, hematopoietic stem and progenitor cells (HSPC) in the peripheral blood of beta-actin/GFP mice [32] did not migrate into the brain of C57BL/6 partners over the course of 14 days [33].

In view of bone marrow transplantation experiments, other groups had hypothesized that the microglia cell population might be replenished at a slow rate by bone marrow derived progenitors, which migrate over the circulation to the brain [10] and that, in this way, a slow turnover of microglia might take place [2].

Dynamics of the healthy Circulation: Migration of cells between organs is often approached by the idea of migration equilibrium [33-35]. This circulation, in the healthy state, of course occurs mainly between more ‘open’ organs than the brain. In the healthy mouse brain migration is severely restricted by the tight endothelial wall encasing the brain, the blood brain barrier. That dramatically changes if cell death in the brain occurs (See chapter on diseased state). A plethora of observations allow to roughly determine the dynamics of stem and progenitor generation and migration in the healthy circulation.

Parabiotic blood HSPC reached a 40% chimerism in the peripheral blood of the partner and high chimerism in ‘open’ organs like spleen, lung, lymph, liver and kidney [33]. However, only 5% chimerism in bone marrow and almost none in the brain could be detected.

According to van Furth et al. [34], monocytes leave the circulation randomly with $T_{1/2} = 17.4h$. The number of monocytes present in the circulation of a healthy, adult mouse is about $4{,}4*10^5$ or $6.18*10^5$ [34, 36]. In the healthy state about $5*10^5$ promonocytes in total mouse bone marrow generate ca. $0.62*10^5$ cells/h [34].

In the experiment by Massberg et al. parabiotic HSPC showed about 36h retention time in peripheral tissues before they remigrated to the blood. This was determined by disconnecting the parabiotic partners after 14 days and measuring the time course of the drop of chimerism [33].

Such observations give insight into cell turnover in the circulation and tissues, how migration and proliferation behavior changes in disease, injury or age and how transplanted cells will behave. They can be applied to design therapies.

Intrinsic Proliferation: In the healthy, young brain microglia are capable to proliferate locally and do not necessarily require replenishment from circulating progenitors [18, 37]. Also, microglia cell numbers in the brain are regulated by apoptosis [31, 38, 39].

Migration under Diseased Conditions

Migration in the diseased Brain: If microglia are completely activated, they withdraw processes and the whole cell body moves to the site of injury or plaques [9]. This holds also true for cells other than resident microglia. If a hemisphere of the brain is injured by intracerebral injection of LPS, intracerebral MSC migrate to the site of injury and proliferate locally [40].

The removal of 'normal' debris takes place without full activation or strong movement of the cell body (See section on healthy conditions). The role of microglia seems to be dependent on distinct sets of surface receptors involved in phagocytosis [15]. Receptors recognizing microbes, for example toll like receptors (TLR), stimulate a pro-inflammatory response. Microglia activated in this way excrete signals associated with inflammation, like tumor necrosis factor (TNF), interleukin IL-1 and nitric oxide (NO).

When microglia were co-cultured with brain tissue and treated with lipopolysaccharides (LPS) they acted neurotoxic. Contrarily, if they were activated with interleukin 4 (IL-4), the microglia acted neuroprotective in co-culture [15, 41]. IL-4 regulatory macrophages can support organ transplantation [42].

Migration across the BBB: In lesioned brain hemispheres (enthorinal cortex lesion) [31], migration of bone marrow cells to the hippocampus was observed, across the BBB, contrary to the unlesioned state (Fig. 2).

Bone marrow derived microglia can be observed in the brain after systemic transplantation [43]. However, while bone marrow (BM) chimeras have shown BM derived microglia [44], experiments indicate that parabiotic circulating blood is not sufficient and bone marrow is necessary for invasion [18].

Other groups could observe migration of intravenously injected hematopoietic stem cells to the brain in ischemic mice. They differentiated to microglia and reduced infarct size [45].

Migration Signals: The invasion of microglia into the central nervous system during development occurs coincidentally with neuronal cell death [20]. Cell death, disease and injury induce various migration signals. Among others monocyte chemoattractant protein 1 (MCP-1), interferon gamma inducible protein (IP)-10, macrophage inflammatory protein (MIP)-1alpha, MIP-1beta and MIP-2 [46]. These chemokines seem to be produced mainly by resident microglia and astrocytes and provide signals for homing of leukocytes to the site of injury and across the BBB (Ibid.).

It is, therefore, apparent that cell death or a disruption of the BBB might result in migration of cells into the brain. However, it is difficult to elucidate the contribution of chemokines and a possible breakdown of the blood brain barrier to cell migration.

Facial nerve axotomy (FNA) – a lesion that does not perturb the blood brain barrier – does not suffice for migration of bone marrow cells into the brain [17]. The group observed that irradiation is necessary for invasion.

Irradiation leads to endothelial cell death (Apoptosis), changes in gene expression, cell-cell interactions and alteration of the microenvironment [47]. The achieved disruption of the BBB is, for low doses, only transient (Ibid.).

Other groups noted that irradiation is not necessary for invasion. In chimeric mice with green fluorescent protein (GFP) marked bone marrow cells, migration of monocytes from the bone marrow to the brain took place in cases of injury like facial nerve axotomy, fimbria-fornix transsection, cuprizone demyelination and stroke [31].

All of these findings indicate that cells in peripheral blood do not migrate to the brain and that mobilization of bone marrow cells are necessary. Also, that irradiation, certain types of injury (facial nerve axotomy, fimbria-fornix transsection, cuprizone demyelination and stroke) and the associated migration signals are necessary conditions for migration of such cells to the brain.

Dynamics of the diseased Circulation: Already hypoxia and stress suffice to mobilize cells into the circulation (Zhang, 2009). Also cytokines like GM-CSF can mobilize HSC into the bloodstream [32] Mimicking mild inflammation of the lungs or peritoneal cavity with LPS or infection, both intranasally and intraperitonally, a decrease of circulating monocytes was observed by Burke et al. [36]. This decrease was interpreted as recruitment of circulating monocytes to the infected site.

Under more serious or acute conditions, for example stroke, the circulating monocyte count increases, however lymphopenia is observable [48]. In such acute cases increased systemic proliferation and, in the case of stroke, immunodepression [49] form a more complex interplay than in mild inflammation.

Intrinsic Proliferation: If an injury is caused through facial nerve axotomy, initially local neuroinflammation and enhanced proliferation of microglia take place. The regeneration of axons is completed over a time frame of about 4 weeks. At this time, the microglia activity has already ceased [10].

Migration from the Brain: After brain invasion by circulating monocytes in the case of a lesion [31], numbers of microglia/macrophages decrease to normal levels possibly by apoptosis and migration out of the brain [38, 39]. It has been proposed that this might involve presentation of antigens in lymph nodes [50]. GFP monocytes migrated from the intracerebral lesion site to deep cervical lymph nodes in 7 days [51]. Such behavior corresponds to the normal migratory circulation of HSPC in more ‚open’ organs [33].

Migration in Age

Migration in the Brain: The functions of microglia like phagocytosis, oxidative burst and cytokine production are impaired in age [10, 14-16, 52-54]. This seems to be partially due to a deterioration of the cells themselves, but also caused by the aged environment. In transplantation experiments, old donor leukocytes performed worse than young did and young leukocytes performed worse in old hosts [55].

Migration across the BBB: It is a well studied fact that vessels become more permeable in age (Fig. 2) [56, 57]. Furthermore, there is evidence that the regeneration of aged vessels by proliferation and endothelial progenitor cells is impaired [58]. BBB permeability increases with normal aging [56, 57]. This might lead to diffusion of harmful substances from the blood into organs and to a constant inflammation throughout the body in old age [56, 57]. It might also contribute to the incidence of neurodegenerative diseases, caused by a chronic activation of immune cells. Other potential reasons of this observed „Inflamm-Aging“ are antigens and stress [59].

Such a porous blood brain barrier, but also an inflamed brain, might change the migration behavior of cells across the BBB in age. Dauer et al. compared migration in 8 week (young) and 52 week (advanced middle age) mice in case of facial nerve axotomy and observed differences in the invasion of T cells into the brain [60]. This was mainly attributed to possible differences in the inflammatory response in aged animals. Hurley et al. observed similar differences and mentioned that BBB breakdown in age might be underappreciated [61].

Intrinsic Proliferation: Some groups hypothesized that resident microglia reach replicative senescence in old age [10]. This has been linked to the development of neurodegenerative diseases, especially Alzheimer, in humans but not in rodents. It is simply suspected that, in case of short life spans, a replicative senescence and complementary deterioration of the microglia cell population does not take place [10]. In old rats there have been indications that proliferation of microglia after injury is stronger than in young rats [62]. *In vitro*, microglia have been reported to undergo telomere shortening [12]. *In vivo,* however, there are reports of higher telomerase activity and stable telomere length of FACS sorted microglial cells after their proliferation was induced by facial nerve axotomy [63].

<u>Migration in Therapy</u>

Application of Cells: In principle, cells can be delivered via the same routes as microglia progenitors migrate into the brain during development. MSC have been delivered intranasally and migrated into the brain [64]. Intravenous injection of GFP-transgenic bone marrow cells after whole body irradiation resulted in reconstitution of the bone marrow and colonization of the brain [43]. Cells can also be directly injected intracerebrally [40].

Cell Size: During the injection procedure, small bore diameter and pressure of syringes have detrimental effects on cell viability [70]. Larger bore diameter reduces these effects. Cells that remained for a longer time in syringes showed reduced viability while cells at room temperature fared better. Cells stored at 4°C performed worse than cells in the syringe (Ibid.).

In the case of intravenous injection, but also on other application routes, cell size strongly influences migration behavior. Especially larger cells, like mesenchymal stem cells (MSC), are often stuck in the lungs for a certain time, resulting in respiratory problems and even death [71]. In fact, minutes after intravenous injection, MSC are mostly situated in the lungs. Then they relocate to other organs, for example to liver and bone marrow [72]. Therefore, on the way to the target organ, a large amount of cells is stuck, filtered out of the blood stream or simply entering other tissues. Cells injected intravenously do not all – as is often nearly insinuated – home directly to a certain organ and treat a certain disease. Instead, such an application is obviously an undirected treatment of the whole organism involving migration of cells to a variety of organs, engraftment and other effects. This occurs especially in the absence of migration signals caused by lesions, infections or degeneration.

Cell Type: Ajami et al. observed that parabiotic blood cells do not suffice, even if the parabiotic partner was irradiated, to observe migration to the brain and that bone marrow is necessary [18, 65]. In this way the group showed that certain bone marrow resident stem or progenitor cells might have the ability to migrate into the brain. At the same time, differentiated cells might be unsuited for migration into the brain (Fig. 3).

Various cell types display the ability to ‘home’ to certain associated organs or sites of injury. The transplanted cell type can, therefore, have an impact on the successful migration into the brain. The developmental migration of microglia fueled the hypothesis of a directed homing of microglia progenitors to the brain [66]. In the case of intravenous injection, epidermal neural crest stem cells and bone marrow derived MSC, when LPS, injected intracerebrally, induced demyelination, migrated to the site of injury (8% of injected cells) [40]. HSPC did not migrate to the brain [33]. Lymphocytes are known to migrate to lymph nodes and spleen [67].

Treatment of Cells: Before transplantation, cells are often treated in various ways. Cell culture, simple exposure to oxygen or enzymatic treatment can have drastic effects on migration properties, homing, cell survival and differentiation.

Homing properties of bone marrow cells are destroyed by prolonged *in vitro* expansion [68]. It is known that fresh bone marrow is generally better suited for reconstitution of the immune system than bone marrow cells which have been in culture over longer periods. Stem and progenitor cells differentiate because of exposure to oxygen, adherence or culture density [69]. However, such *in vitro* cultivated BM cells are not only less suited for reconstitution but also have a highly reduced ability to reach spleen or bone marrow [68]. In a similar way, possible special migration properties of microglia progenitors might be compromised [66].

Trypsinization destroys surface adhesion molecules and therefore will restrict cell adherence and directed migration [67]. After treatment of lymphocytes with trypsin for 5 minutes, they had lost their ability to enter lymph nodes but could still enter the spleen (90 min after injection). When analyzed 16 h after injection, lymphocytes could again be found in lymph nodes and spleen. This suggested a regrowth of the necessary adhesion molecules over a longer time period [67].

Dynamics of Transplanted Cells in the Circulation: Over a course of 2-7 days in a healthy mouse, injected MSC enter various tissues like liver or bone marrow [72]. This corresponds to theoretical considerations. Under normal conditions, monocytes leave the circulation randomly with a half time of 17.4h [34]. For example, 10^6 monocytes would, together with the circulating and marginating cell pool of $1.5*10^6$ [34], result in a transient doubling of circulating monocytes. From these models, after 1 day a 25% chimerism remains in the circulation. After 1 week less than 0.1%.

Migration Signals and Permeabilization of Blood Brain Barrier: The comparison of cell migration under healthy and diseased conditions shows that the induction of certain injuries or irradiation is necessary to induce migration to the brain. Facial nerve axotomy does not suffice to induce migration of bone marrow cells and irradiation of the brain is necessary [18]. Fimbria fornix transsection, cuprizone demyelination and stroke result in migration of monocytes from bone marrow into the brain [31]. Intracerebral LPS draws epidermal neural crest cells and bone marrow derived MSC into the brain [40].

Acute injuries like stroke or even irradiation do not just result in neuronal death and chemokines but also disrupt the blood brain barrier. Such an indiscriminate disruption is dangerous as it not only damages the brain but also allows the diffusion of harmful substances into the brain.

Cell Survival, Dose and Rejection: Balb/c SR/CR leukocytes convey resistance to cancer. When female Balb/c SR/CR donor leukocytes were injected intraperitonally into male Balb/c hosts, their survival was not affected by sex mismatch [55]. Survival of the cells was tested rather robustly by challenging the hosts with S180 cancer cells, 1 day to 12 weeks after transplantation, and measuring the rate of survival of the hosts. Male Balb/c SR/CR donors into female hosts showed only 83% survival. MHC mismatch resulted in 58% survival and added with female mismatch (43%). Old donor leukocytes resulted in lower survival of host mice than young did and young leukocytes archived lower survival in old hosts [55].

Stewart et al. hypothesized a ‘competitive engraftment’ [35]. In non-irradiated conditions injected and resident bone marrow cells compete for stem cell niches in the bone marrow. The group reported that, without irradiation, large cell doses (40-200*10^6) of male Balb/c, BDF1, CBA-J mice into matched female hosts could result in high and stable (1 year) chimerisms of 30%-70%. With BMT, already doses of 5*10^6 cells achieve a similar 40%-60% chimerism [28]. In line with this, Massbergs parabiosis experiment resulted in a comparatively small 5% chimerism of HSPC in the non-irradiated, matched partner bone marrow [33].

***In vivo* Proliferation:** *In vivo*, proliferation induced by inflammation or irradiation can drastically influence the outcome of a therapy. Mildner et al. irradiated the mouse body excluding the brain, thereby inducing inflammation. However, cells could not be observed to enter the non-irradiated brain, rendering the contribution of *in vivo* proliferation to migration into the brain questionable [17].

Competing Interests

No competing interests.

Authors' Contributions

AH wrote the manuscript. AS contributed to writing the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Figure 1: Natural migration routes in and out of the brain during development and possible application routes in a cell therapy. MP: Microglia progenitors, HSPC: Hematopoietic stem and progenitor cells.

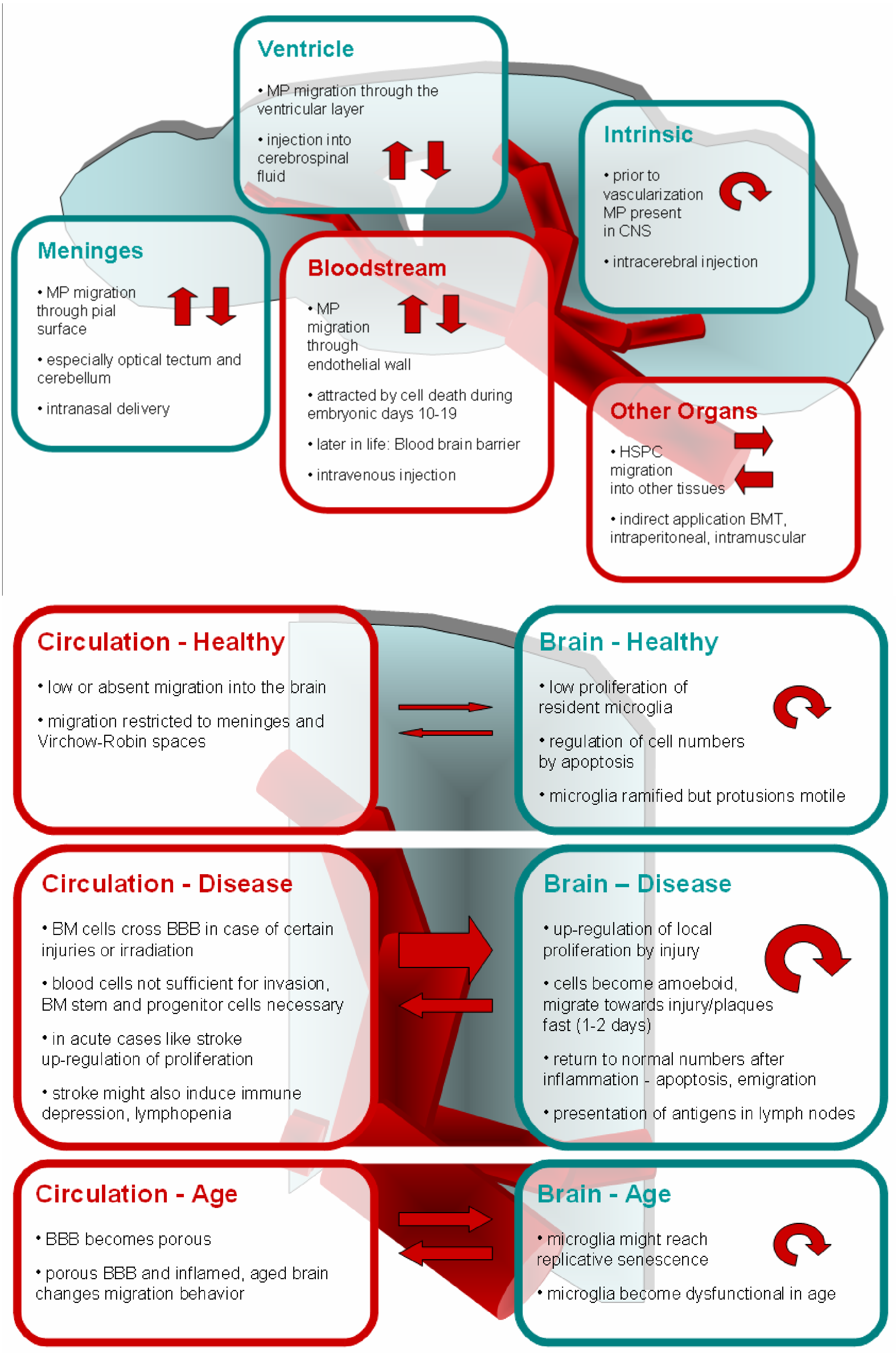
Figure 2: (A) Migration under healthy conditions. The brain is shielded by a tight endothelial barrier and migration in and out of the brain is low. (B) Diseased or injured conditions change migration behavior. Certain injuries prompt bone marrow (BM) cells to migrate into the brain. Acute injury also up-regulates proliferation and might result in immunodepression. (C) In old age the endothelial wall becomes porous and a general inflammation of organs results.

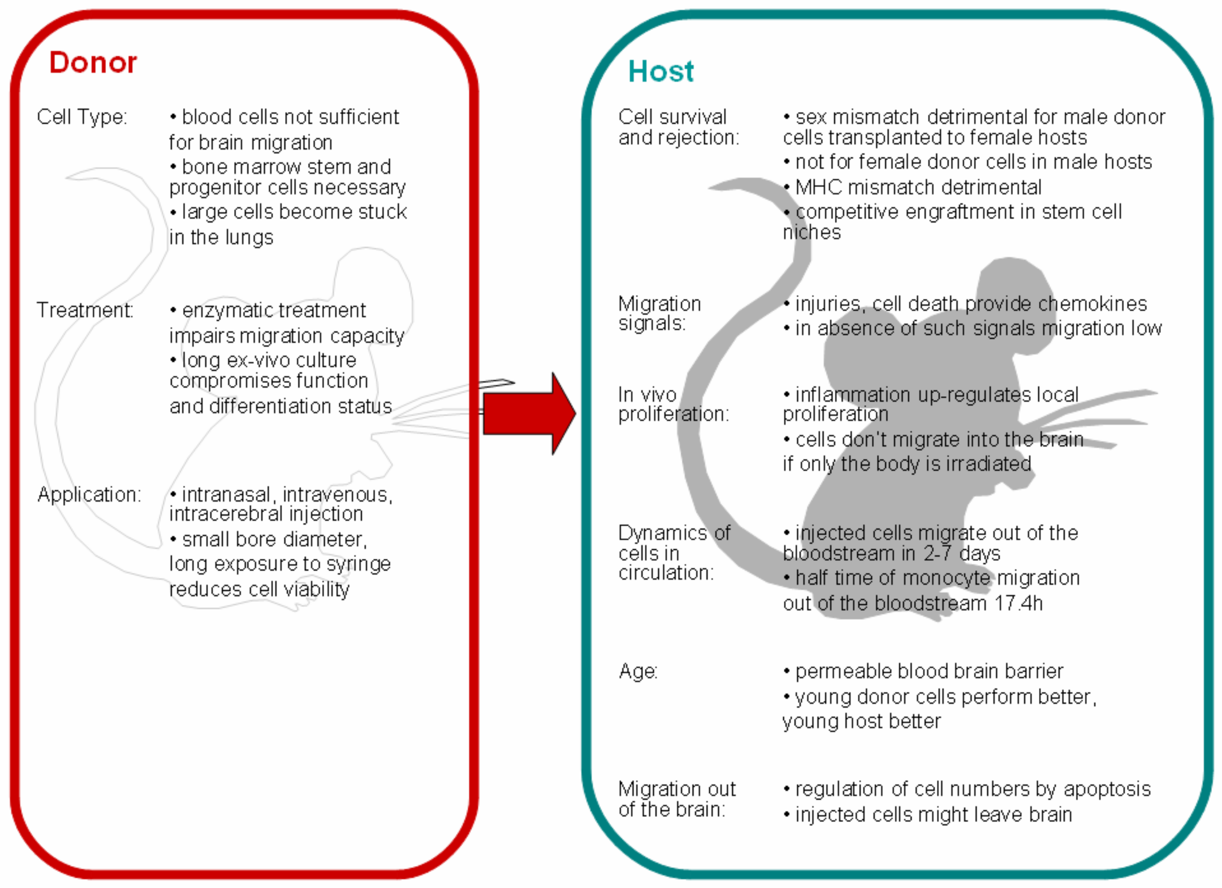
Figure 3: Important therapeutic parameters that influence the migration of injected cells. In absence of migration signals due to disease or injury cell migration into the brain is low. Also, cell migration inside the brain is limited.

Table 1:

Sources that investigate important Migration Parameters.

Migration Parameter	**Investigated in**
Cell Type	Imai 1997 Ajami 2007 Massberg 2007
Treatment of cells (Trypsinization, cultivation)	Nolte 2001 Szilvassy 1999
Application	Danielyan 2009 Rodriguez 2007 Jackson 2010 Agashi 2009
Cell survival, dose, rejection	Stehle 2009 Steward 1993 Bechmann 2005 Coyne 2006
Cell size	Gao 2001 Schrepfer 2007
Permeabilisation of the blood brain barrier	Gaber 2004 Mildner 2007
In vivo proliferation	Mildner 2007
Dynamics of transplanted cells in circulation	Van Furth 1986 Massberg 2007
Age	Farrall 2009 Valle 2009
Migration out of the brain	Kaminski 2012 De vos 2002 Finsen 1999


Ventricle
• MP migration through the ventricular layer
• injection into cerebrospinal fluid
Intrinsic
• prior to vascularization MP present in CNS
• intracerebral injection
Meninges
• MP migration through pial surface
• especially optical tectum and cerebellum
• intranasal delivery
Bloodstream
• MP migration through endothelial wall
• attracted by cell death during embryonic days 10-19
• later in life: Blood brain barrier
• intravenous injection
Other Organs
• HSPC migration into other tissues
• indirect application BMT, intraperitoneal, intramuscular
Circulation - Healthy
• low or absent migration into the brain
• migration restricted to meninges and Virchow-Robin spaces
Brain - Healthy
• low proliferation of resident microglia
• regulation of cell numbers by apoptosis
• microglia ramified but protusions motile
Circulation - Disease
• BM cells cross BBB in case of certain injuries or irradiation
• blood cells not sufficient for invasion, BM stem and progenitor cells necessary
• in acute cases like stroke up-regulation of proliferation
• stroke might also induce immune depression, lymphopenia
Brain – Disease
• up-regulation of local proliferation by injury
• cells become amoeboid, migrate towards injury/plaques fast (1-2 days)
• return to normal numbers after inflammation - apoptosis, emigration
• presentation of antigens in lymph nodes
Circulation - Age
• BBB becomes porous
• porous BBB and inflamed, aged brain changes migration behavior
Brain - Age
• microglia might reach replicative senescence
• microglia become dysfunctional in age


Donor
Cell Type:
• blood cells not sufficient for brain migration
• bone marrow stem and progenitor cells necessary
• large cells become stuck in the lungs
Treatment:
• enzymatic treatment impairs migration capacity
• long ex-vivo culture compromises function and differentiation status
Application:
• intranasal, intravenous, intracerebral injection
• small bore diameter, long exposure to syringe reduces cell viability
Host
Cell survival and rejection:
• sex mismatch detrimental for male donor cells transplanted to female hosts
• not for female donor cells in male hosts
• MHC mismatch detrimental
• competitive engraftment in stem cell niches
Migration signals:
• injuries, cell death provide chemokines
• in absence of such signals migration low
In vivo proliferation:
• inflammation up-regulates local proliferation
• cells don't migrate into the brain if only the body is irradiated
Dynamics of cells in circulation:
• injected cells migrate out of the bloodstream in 2-7 days
• half time of monocyte migration out of the bloodstream 17.4h
Age:
• permeable blood brain barrier
• young donor cells perform better, young host better
Migration out of the brain:
• regulation of cell numbers by apoptosis
• injected cells might leave brain

6. Transplantation and Stereological Measurements

Stereological Measurement of Microglia Activity in the Mouse Brain

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Abstract

The brain changes with age and many diseases of the brain are in fact age related. Especially the function of the immune system and here of microglia has been linked to diseases developing in old age.

To elucidate the causes and develop therapies the establishment of robust and dependable methods to measure such age related changes are important. Here we suggested the application of stereological methods to measure changes in microglia activity with age, so called inflamm-aging.

We describe advantages and limiting problems of the method that will be useful to anyone applying stereology to biological samples.

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Key words:

Introduction

Age related diseases of the brain have been linked to the deterioration of microglia in age. Among other changes, deterioration of cells [1], a loss of function [2-5] and chronic activation [6] have been observed. We wanted to investigate here changes in activity of microglia with age. This has been done previously using non-stereological methods [7]. Measuring microglia ‘activity’ using immunohistochemical and stereological methods presents several problems, most prominently the definition of microglia activation but also variations in the investigated regions, time consumption, problems involving the staining of cells and statistical considerations.

Materials and Methods

Animals

C57BL/6 mice from the animal facility (MEZ) of the University of Leipzig and Charles River (Sulzfeld, Germany) were used to create brain slices in accordance with local animal ethics regulations. Two groups were defined, a young (2-3 months) and an old (12 months) group.

Brain isolation and cryoslices

The mice were perfused with 4% paraformaldehyde (PFA) and the brain isolated. The brain was placed into 4% PFA for 1 day. Afterwards the brain was moved to 30% sucrose/0.1% NaN_3 for 1-2 days. The brains were snap frozen in methylbutan. They were stored in a –80°C freezer together with some ice.

The brains were moved from the –80°C freezer to –20°C 24h before they were cut in a cryostat. The part covering the hippocampus was cut in a cryostat CM3050S (Leica) in 40 µm slices. The slices were, using a fine brush, placed in a 48 well plate filled with cryo protection solution (CPS). The CPS consisted of 250 ml glycerin, 250 ml ethylenglycol and 500 ml DPBS.

Immunohistology

The brain slices were stained over night (4°C) with mouse x MHCII (mouse, rat), 1:500 dilution (Abcam, ab23990) and rabbit x Iba-1, 1:500 dilution (Wako, 019-19741). They were stained for 2h in the dark with the secondary antibodies goat x mouse PE, diluted 1:1000 (Abcam, ab7002) and donkey x rabbit Cy2 1:1000 (Jackson Immunology, 711-225-152). Afterwards, the slices were stained with a 1:30000 solution of DAPI (Sigma Aldrich, D9564-10MG) and incubated in the dark for

20 min. The slices were placed in a box with CPS and washed onto glass slides with a fine brush, dried over night, mounted with DPX and dried for additional 24 h.

Preliminary considerations

The stereology system used was the Stereoinvestigator (MBF Bioscience). For stereology based studies statistical considerations play an important role to reduce the resulting error. The coefficient of error (CE) gives an estimate how large the contribution of the stereological estimation to the resulting statistical error is. To obtain a low overall error the CE should be dominated by the statistical error among animals. We stained every 8^{th} slice, covering the hippocampus [8]. Lower intervals lower the estimation error, however do also consume more time for slice preparation and counting. The interval is sufficient in our case (n=3) to ensure that the error of stereological estimation is dominated by the variation between animals. If more animals are measured additional slices can be added (For example every 4^{th}, including the previously counted).

Frame and grid size (80µm x 80µm, 250µm x 250 µm) were chosen such that about 600 - 800 cells per brain were counted. Like the interval between counted slices, larger numbers of counted objects lower the error of estimation and should always be chosen such that the resulting error of the estimation is dominated by the variation between animals.

Results and Discussion

Counting region definition

Because biological samples are very variable one of the major error sources lies in the difficulty to objectively define a region in which to count cells (or other objects). This is especially problematic because of the easy shrinking/shearing of brain slices or other biological samples.

Simply applying a box of a certain size to a random part of the 3-dimensional hippocampus – as often done - will clearly result in errors because both varying regions are measured but also the box would span regions that were originally of larger or smaller size. Such errors would partly be systematic, i. e. be observed as significant differences that do not truly exist. However, such problems can partially be ameliorated by calculating densities instead of using absolute numbers or volumes.

The definition of a region in a biological sample should in the best case scale with shrinking/expanding or shearing. Furthermore, the region should be defined 3-dimensional.

We have used here the easily identifiable outlines of Dentate Gyrus and Cornu Ammonis to define a 3-dimensional volume in which to count (See Figure 1A). This definition accounts for shrinking or shearing. We count these regions in both hemispheres to avoid problems from accidentally mixing up the right and left hemisphere. Furthermore, in this region microglia are under non-pathological conditions evenly distributed. Such even distribution simplifies the counting in biological samples. Cells which are strongly accumulating in single places are often impossible to count.

However this definition has also several drawbacks. In figure 1A it is possible to see that the ‘rear’ end of the region is open and the Cornu Ammonis becomes diffuse there. This might result in variation of the volume of the region. The volumes we determined, however, did change only very slightly between animals (Fig. 3C). Therefore, the inner hippocampus region marked by the Cornu Ammonis and Dentate Gyrus turned out to be well suited for an objectively defined region.

Furthermore, the dense cell accumulations in the Cornu Ammonis/Dentate Gyrus interfere with immunohistology (see section on immunohistology). We used DAPI (1:10000-1:30000) to identify the structures of Dentate Gyrus and Cornu Ammonis.

Immunohistology

Depending on whether ‘microglia’ are defined as cells expressing Iba-1, other markers, expressing them strongly or expressing them at all, or by different methods, this might result in under or over estimation of microglia numbers.

As marker for microglia we used Iba-1. It stained both processes and microglia well (Fig. 1B). Alternative markers would be CD11b, F4/80 and lectin.

A problem arising in the stereological counting of microglia is the relatively continuous expression levels of Iba-1 in different microglia which results in a rather subjective cut off at which low Iba-1 staining is judged as not any more indicative of microglia. This subjective threshold will change between different persons counting cells and result in systematic errors.

As marker for ‘activity’ of microglia we used MHC class II (Fig 2A, B, C, D) as done by Sheffield et al. [7]. MHC class-II is involved in phagocytosis and marks cells which are highly phagocytic [9]. Microglia expressing MHC class-II have been observed to be hightly phagocytic while MHC-II negative microglia are not.

Therefore, we define here ‘active’ microglia as MHC-II expressing, phagocytic microglia. One might argue that microglia activation from their ramified, resting state is not necessarily identical with increased phagocytosis – although many studies reported increased phagocytosis in activated microglia populations.

There are microglia that strongly express or do not express at all MHC-II (Fig. 2A, B, C, D). However, most ‘active’ cells are intermediate stages of these extremes and express MHC-II only on certain spots. MHC-II is often expressed on the processes of microglia (Fig 1D) and not only on their cell body. Most of the time it is not possible to clearly distinguish which protusion corresponds to which microglia, especially because these protusions form a three dimensional network. Therefore we defined here ‘active’ microglia as those which express MHC-II on their cell body, not on their protusions. This definition is clearly motivated by the method and not at all biologically. It might result in systematically counting lower numbers than actually active microglia (Since all those which do only express MHC-II on their protusions are counted as ‘resting’). Furthermore, there might be biological reasons why some microglia express MHC-II on their protusions and others on their immediate cell body. MHC-II on the cell body is sometimes expressed on the whole body (Fig 2A, B) but in most cases only on a part (Fig. 2C, D). Often, MHC-II is observable at the spot where a protusion connects to the cell body (Fig. 2G, H, I, J)

Also, other markers have been used in the past to differentiate between ‘active’ and resting microglia without observing cell morphology (ramification). Most of them, however, have been reported to be gradually up-regulated (CD45, CD68) which makes a clear distinction between ‘active’ and resting difficult.

Furthermore, the high cell density in the Dentate Gyrus and Cornu Ammonis regions often resulted in strong MHC-II staining in these regions. In these parts of the hippocampus it is difficult to determine whether the MHC-II is really expressed by the Iba-1 positive microglia in question or lying on top or below the cell.

Microglia numbers and activity

We could observe significant differences in microglia activity between young (2-3 months) and adult (12 months) mice (Fig. 3D). Sheffield et al. showed a significant increase of MHC-II in normal brain aging of rodents and primates [7] Also microglia numbers are significantly different – however, when the density is calculated this vanishes (Fig. 3A, B). There have been reports of rising microglia numbers in female mice [10]. However, here variations in the volume of the counting regions are responsible for this.

Coefficient of error

When on every 8th slice hippocampi of both hemispheres were counted, the coefficient of error of the stereological estimation (CE) was always at or below 0.05. Compared to the resulting statistical errors between samples, this error of estimation did not dominate the errors by animal variation or immunohistology. For example, the absolute microglia numbers determined for young animals were 67490. The resulting standard error was 6106. On the other hand, the CE value in these 3 cases was 0.05, 0.05 and 0.04. The resulting error of the estimation was therefore 3375. As is desired, the CE value was dominated by variations between animals.

For calculation of the CE (coefficient of error) we used here the error estimation of Gunderson with the assumption of m=1 [11] – assuming that the counted volume and the counted microglia are smooth and evenly distributed. Use of m=0 results in a slightly higher CE (<0.06) that is, however, still dominated by the statistical error. If more animals are counted, the intervals of counted slices and grid size would have to be lower for best statistical results.

Outlook

Since robust measurements of age related changes are important for their investigation and for the development of therapies, the reproduction of the here reported significant differences in microglia activity by other groups would be important. Also, since the age difference is low (young and adult mice), an extension to older mice and also other species would be interesting.

MHC-II, used here as activity marker has certain advantages and drawbacks over other markers. It would be advantageous to compare different markers that are clearly expressed on active, but not on resting, microglia and to find markers which are exclusively expressed on the cell body and better suited for the highly dense Dentate Gyrus/Cornu Ammonis.

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We want to thank Antje Rückner for immunohistology of the brain slices.

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Figure legends

Fig. 1: (A) The hippocampus region was defined by the outlines of the Cornu Ammonis and Dentate Gyrus. **(B, C)** Representative pictures of microglia in brain slices stained with Iba-1 (Green). (**E)** Overlay of **(C)** with red (MHC class II) channel **(D)**. MHC class II is co-localized with microglia processes and the microglia cell body.

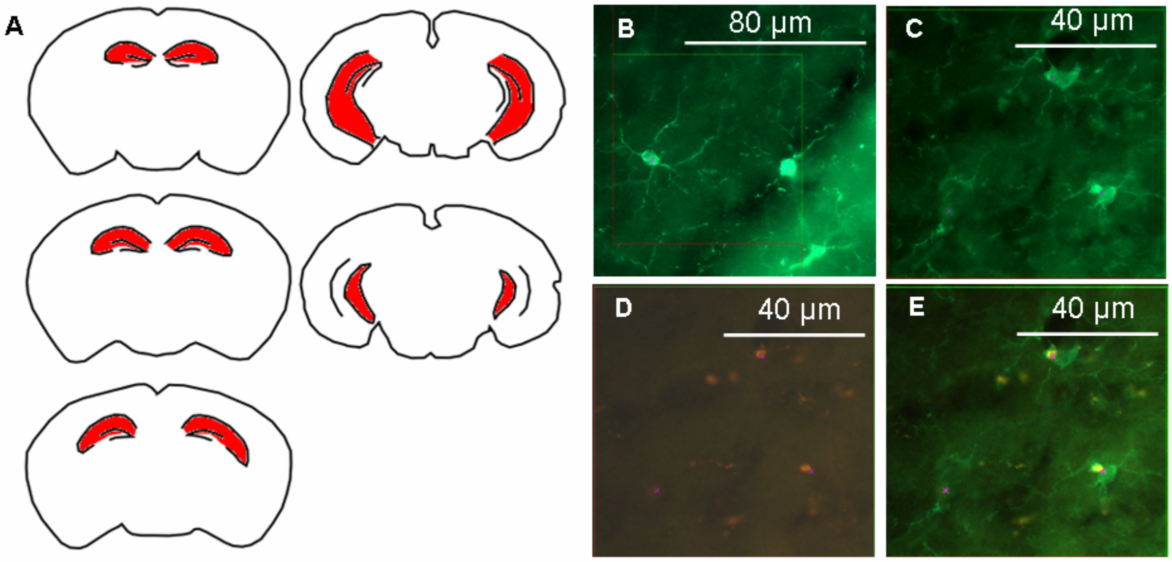
Fig. 2: (A, B) Example picture of a microglia cell body completely covered with MHC class II (‘active’) (MHC class II red, Iba-1 green). **(C, D)** Example picture of a microglia cell body completely devoid of MHC class II (‘inactive’). **(E-J)** Examples of

intermediates (‘active’). MHC class II is often expressed at the link between processes and cell body.

Fig. 3: (A-D) Stereological estimation of absolute numbers of microglia in hippocampus, hippocampus volume, microglia density and activity in young mice (‘Young’, 2-3 months) and adult mice (‘Adult’, 12 months) (*n* = 3). Error bars shown are standard error (SE) of the mean. Statistical analysis by ANOVA: *** = P < 0.001, ** = P < 0.005, * = P < 0.01

Fig. 4: (A-D) Effect of MSC transplanatation. There is a non-significant probability (0.08) that MSC transplantation influences microglia activation. (‘Young’, 2-3 month mice) (‘Adult’, 12 month mice) (*n* = 3). Error bars shown are standard error (SE) of the mean. Statistical analysis by ANOVA: *** = P < 0.001, ** = P < 0.005, * = P < 0.01

Fig. 5: (A-D) NA-BMC transplantation does not influence microglia numbers and activity in the hippocampus. (‘Young’, 2-3 month mice) (‘Adult’, 12 month mice) (*n* = 3). Error bars shown are standard error (SE) of the mean. Statistical analysis by ANOVA: *** = P < 0.001, ** = P < 0.005, * = P < 0.01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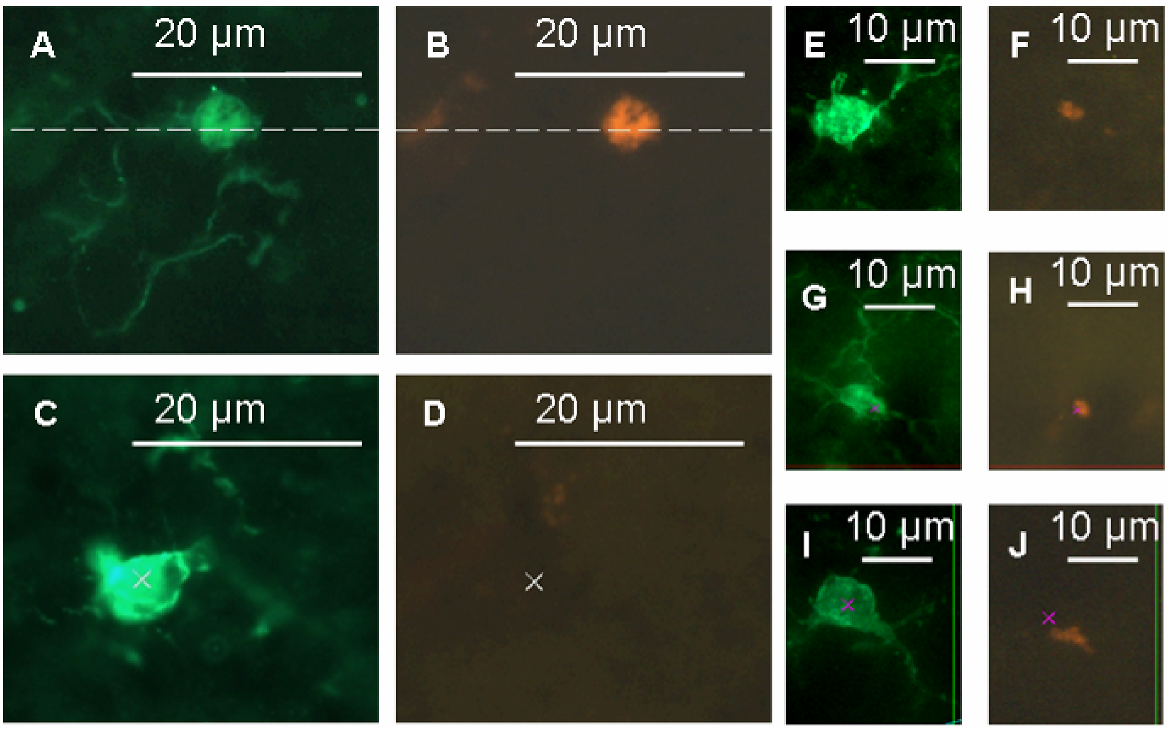
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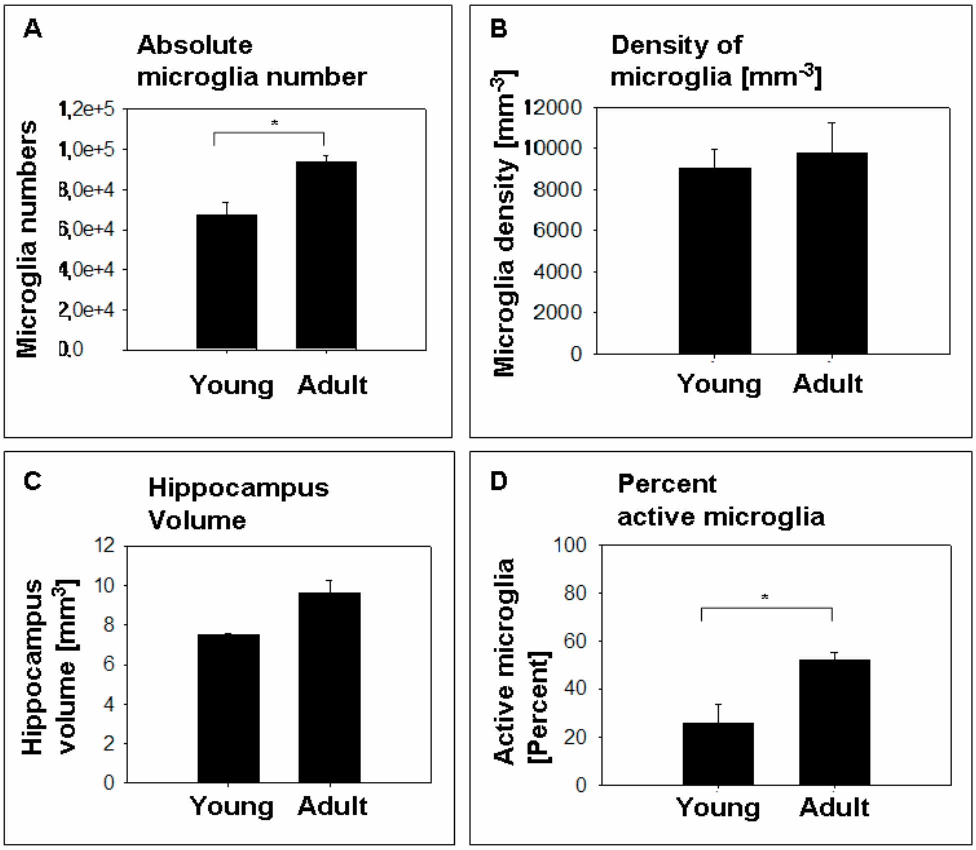
A
Absolute microglia number
Microglia numbers
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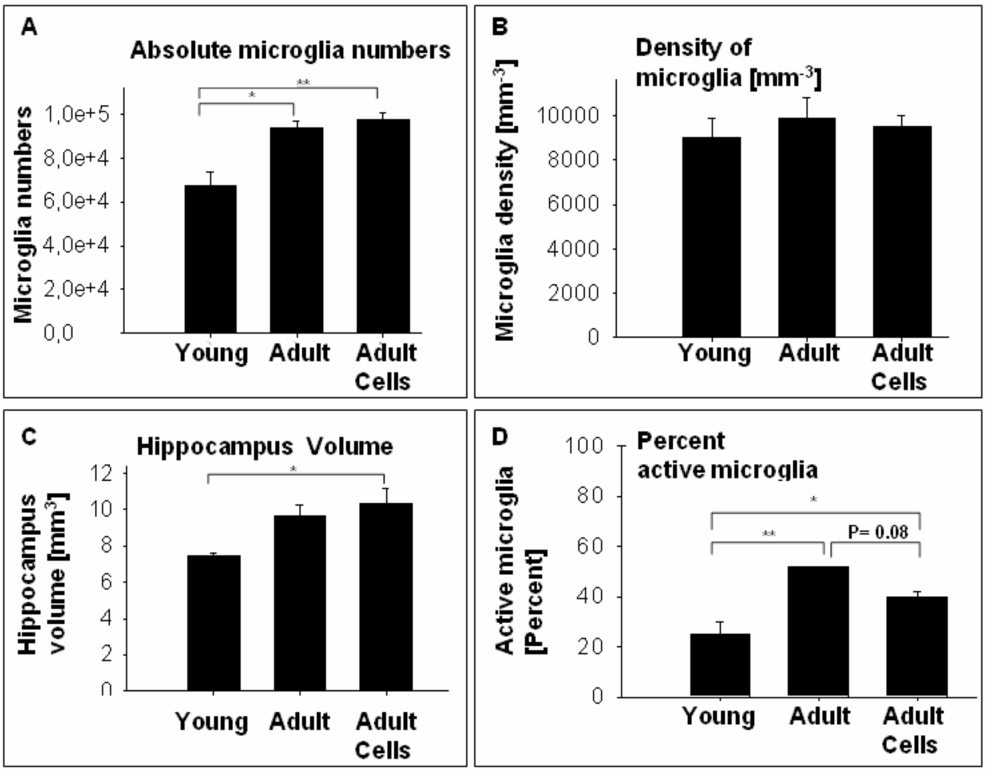
1,0e+5
8,0e+4
6,0e+4
4,0e+4
2,0e+4
0,0
*
Young
Adult
B
Density of microglia [mm-3]
Microglia density [mm-3]
12000
10000
8000
6000
4000
2000
0
Young
Adult
C
Hippocampus Volume
Hippocampus volume [mm3]
12
10
8
6
4
2
0
Young
Adult
D
Percent active microglia
Active microglia [Percent]
100
80
60
40
20
0
*
Young
Adult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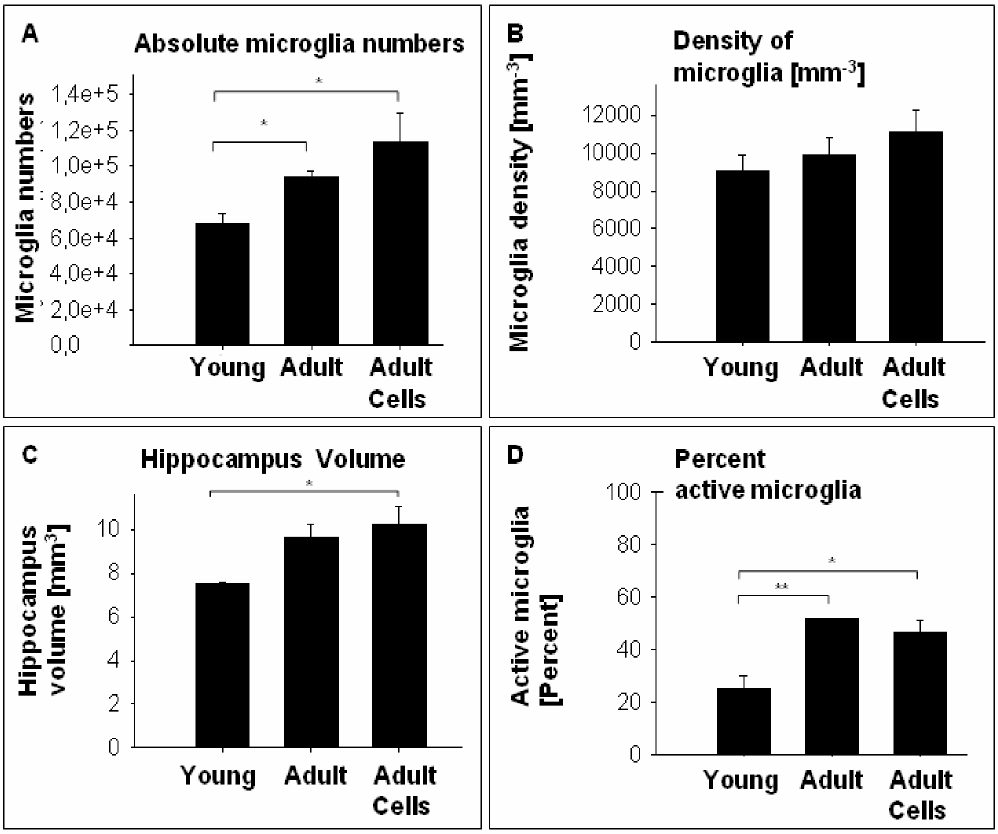
Absolute microglia numbers
Microglia numbers
1,0e+5
8,0e+4
6,0e+4
4,0e+4
2,0e+4
0,0
*
**
Young
Adult
Adult Cells
B
Density of microglia [mm-3]
Microglia density [mm-3]
10000
8000
6000
4000
2000
0
Young
Adult
Adult Cells
C
Hippocampus Volume
Hippocampus volume [mm3]
12
10
8
6
4
2
0
*
Young
Adult
Adult Cells
D
Percent active microglia
Active microglia [Percent]
100
80
60
40
20
0
*
**
P= 0.08
Young
Adult
Adult Cells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Absolute microglia numbers
Microglia numbers
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1,2e+5
1,0e+5
8,0e+4
6,0e+4
4,0e+4
2,0e+4
0,0
Young
Adult
Adult Cells
B
Density of microglia [mm-3]
Microglia density [mm-3]
12000
10000
8000
6000
4000
2000
0
Young
Adult
Adult Cells
C
Hippocampus Volume
Hippocampus volume [mm3]
10
8
6
4
2
0
Young
Adult
Adult Cells
D
Percent active microglia
Active microglia [Percent]
100
80
60
40
20
0
Young
Adult
Adult Cells

7. Discussion

Aims

Alzheimer or Multiple Sclerosis might be connected to a chronic activation or loss of function of microglia in age [1]. Functional microglia differentiated from adult stem cells might be transplanted to resolve these problems.

We aimed to:

1. Differentiate microglia from adult stem cells (mouse bone marrow) and proof their function (**Paper 1 and 2**)
2. Observe migration, survival and microglia morphology in co-culture with brain tissue (**Paper 1 and 2**)
3. Transplant microglia and MSC, track cells and stereological measurement of microglia numbers and activity (**Paper 3 and additional data**)

Differentiation of Microglia

Microglia differentiation: We screened several protocols for differentiation of functional microglia from bone marrow (Data not shown). A number of these protocols resulted in cells showing markers, morphology and function of primary microglia (**Paper 1 and 2**).

To date, several protocols to differentiate microglia have been reported. Peripheral blood monocytes, bone marrow, bone marrow subpopulations and ESC have been differentiated to microglia [4-7].

It was interesting to note that in all of these protocols populations of non-adherent cells are employed which are then differentiated either in ACM or with cytokines. The various reported protocols differ mainly in the *in vitro* culture time before non-adherent cells are selected. Also, they differ in differentiation times and in culture densities and media composition. Especially the differences in media and culture densities make it hard to directly compare the protocols of different groups to each other.

We used GM-CSF and ACM as opposed to ACM alone [6] or M-CSF used by Davoust et al. [5]. GM-CSF has been reported to expand primary microglia more successfully than M-CSF [9, 71].

The differentiation to microglia has, to date, been judged using non-exclusive markers and morphology by other groups [6]. Primary microglia have been characterized as CD11b$^+$/CD45low and have been discerned from primary macrophages by their morphology and CD45 expression level [66, 67].

Markers: Our BM derived microglia showed similar expression levels of CD11b, CD45 and F4/80 as primary microglia (**Paper 1 and 2**). However, also a bone marrow subpopulation shows similar marker expression. It is, therefore, difficult to assess microglia differentiation by markers alone.

Morphology: Microglia differentiated in presence of ACM/GMCSF but without Flt3L displayed a strong ramification as is typical for microglia [6, 30, 31]. The majority of these cells were ramified, pointing towards a high purity of the non-adherent cells of day 11 bone marrow culture. Servet-Delprat et al. observed 20% of differentiated cells to be ramified [6].

Function: Bone marrow cells cultured in the presence of ACM, GM-CSF showed microglia-typical phagocytosis and oxidative burst activity. ACM contains M-CSF and GM-CSF and has repeatedly been used to induce differentiation (ramification) of monocytes and macrophages to microglia.

Flt3L: Flt3L supplementation diminished the functional markers and microglia like morphology. Therefore, among the parameters tested here, the ‘optimal’ protocol for *in vitro* differentiation of microglia relies on ACM, GM-CSF without Flt3L. Flt3L has been used for the sequential differentiation of BM cells presumably because it improves hematopoietic stem cell (HSC) survival *in vitro* [6] and *in vivo* [72].

Servet-Delprat et al. focused on Flt3L supplemented cells and did not delve into un-supplemented cells. The group estimated 20% microglia from the number of ramified cells, which is confirmed by our results for ACM, GM-CSF, Flt3L supplemented cells. However, much higher microglia ‘yield’ can be obtained in the absence of Flt3L. In fact, we demonstrate that supplementation with Flt3L diminishes microglia differentiation: where Flt3L is added by itself or in combination with ACM, GM-CSF, the number of cells showing microglia markers, as well as the capacity for brain migration, phacocytosis and oxidative burst decreases. The differentiation protocols investigated here rely on using the supernatant at day 11 to select for non-adherent progenitor cells, which are then cultured in the presence of ACM for another 6 days. The tactic is to first obtain a relatively pure progenitor population, which then differentiates in part into adherent microglia. Flt3L has been shown to expand HSC and to transiently increase adhesion of HSC in culture. It might play a role in mobilization of HSC to the blood stream [73]. Therefore the amount of microglia progenitor cells in the supernatant of the 11th day bone marrow culture might be decreased. In addition, Flt3L combined with GM-CSF has been shown to enhance dendritic cell differentiation [72, 74]. These factors may explain why Flt3L supplementation yields a lower fraction of functional *in vitro*-derived microglia.

Co-culture with Living Brain Tissue

Co-culture: Microglia derived from adult stem cells have to be able to migrate, survive and integrate into living brain tissue. In pre-transplantation studies we checked these functions by co-culturing differentiated microglia of several protocols with organotypic hippocampal brain slices (**Paper 1 and 2** and data not shown)

Survival: We could show here that our differentiated microglia survived and proliferated in co-cultures with organotypic brain slices for at least 10 days.

Migration: Most cells migrated into the brain slice tissue superficially, while *in vitro* derived (ACM/GM-CSF, but without Flt3L) microglia migrated deepest into the tissue. It is known that the majority of primary microglia or BV2 cells just migrate over the surface layer of brain tissues under non-inflammatory conditions while a subpopulation migrates into the tissue [75-77]. Directed migration towards sites of injury induced by NMDA on the surface of brain slice cultures has been shown for primary microglia [76]. The damaged surface of the brain slice cultures even attracts slice internal microglia showing directed migration to the surface [75].

Morphology: The differentiated microglia in co-culture showed both amoeboid and rounded morphologies, suggesting an activated state. They did not show pronounced ramification after 10 days. It is known that microglia of the brain itself move to the damaged surface after brain slices are cut [76]. It might be suspected that the seeded microglia similarly are activated by the damage and take part in removing debris. This would also explain the round cell shapes in co-culture, which only slowly settle to amoeboid shapes after 10 days.

Phagocytosis: The disappearance of cell debris stained with PI over time might be interpreted as phagocytic activity of the seeded cells. However, it could also be caused by the brains own microglia [75] or simply by medium changes which washed the cell debris away.

Transplantation, Cell Tracking and Stereology

Transplantation: In healthy, young animals migration of cells across the BBB is absent or low. However, it is present in the lesioned, diseased and inflamed brain *[43, 46, 78]*. A permeable blood brain barrier and inflammation in old age might change the migration behaviour in old age [79, 80]. Therefore, we hypothesized that transplantation in diseased or older animals might allow such migration (**Paper 3**).
We transplanted NA-BMC and MSC intravenously in young (2-3 months), adult (12 months) C57BL/6 mice and Alzheimer mice (9 months) (C57BL/6J TgN (Thy1-APPKM670/671NL; Thy1-PS1L166P)).
Cell Tracking: The cells were tracked by RT-PCR based detection of male DNA in tissue samples of sex mismatched hosts. After a follow up of 7 days, NA-BMC, transplanted intravenously in adult (12 months) mice, could be found in bone marrow and lungs but not in the brain and liver. Intravenous MSC could not be detected in bone marrow, liver, lung and brain of adult mice (Data not shown). This cell tracking method is sensitive, however, it is known that under healthy conditions few cells migrate to the brain and cross the BBB (**Paper 3**). Many of such systemically transplanted cells invade other tissues and larger cells become stuck in the lungs – as we did observe. Also, the comparatively low number of transplanted cells in absence of irradiation makes engraftment and detection harder.
Stereology: The transplantation of NA-BMC and MSC did not significantly influence microglia activity in the brain as measured by stereology (**Additional Data**). Since both cell types could not be found in the brain such effects can not be expected or would be indirect. For example, MSC in sufficient numbers regulate the immune system organism wide [3, 21].
The method of microglia activity measurement – co-expression of microglia markers (Iba-1) and activity markers (MHC-II) – has sucessfully been used by other groups [81] but has several drawbacks. First, MHC-II is often expressed on the protusions of microglia and can, therefore, not clearly be linked to a certain microglia – the method

might systematically under- or overestimate ‘activity’ of microglia. Furthermore, the chosen markers might not completely coincide with the actual population of ‘active’ microglia or only catch one aspect of microglia activation – in the case of MHC-II phagocytosis.

Stereology does, however, avoid many of the pitfalls present in less systematic counting of cells, such as the largely arbitrary choice of the region in which cells are counted or the problem of shrinking, expanding or shearing of biological tissues, The resulting errors are systematic, and therefore would not appear as statistical variation between several measured samples but rather as significant, false positive differences between experimental groups.

Age related Changes: We could, however, observe age related changes in microglia numbers and activity between young and adult C57BL/6 mice (**Additional Data**).

Microglia activity is significantly stronger in adult animals (52% compared to 25% in young animals). This microglia activity was defined after Sheffield et al. [81] as expression of MHC-II on the cell body of the microglia, defined as Iba-1 positive cells. The rise in activity is in line with observations by other groups [81].

The numbers of microglia in previous stereological studies [24, 82] were estimated to be about 37000 in the Dentate Gyrus region. We estimated the number in a region spanning the whole inside of Cornu Ammonis and Dentate Gyrus in both hemispheres. We would, therefore, roughly expect 60000 microglia in one hemisphere and 120000 in both. This is consistent with our results.

We observe an increase of absolute microglia numbers in the hippocampus of adult female animals (93938) compared to young female animals (67490). However, also the size of the hippocampus increases. The difference in microglia number vanishes if the density of microglia is calculated. It might be suspected that the change is caused by normal growth of the hippocampus.

Previous papers investigating the brain using stereological methods have reported an increasing number of microglia in the brain of female, but not male, mice with age. Mouton et al. saw an increase from 55000 to 69000 microglia in a Cornu Ammonis region [24] between young (4-6 months) and adult (13-14 months) female mice. However, the numbers between adult (13-14 months) and old (20-24 months) female mice stayed constant. It has been hypothesized that this is caused by inflammation or sex hormones [24]. Other groups, however, have observed a diminishing effect of estrogen on microglia numbers.

Outlook

Microglia Differentiation: Our results and the results of others show the feasibility of deriving microglia from bone marrow [4-6]. The cells can now be continuously produced using the established optimized protocol.

In vitro differentiated microglia need to be able to perform functions of primary microglia. We proved here the function (Phagocytosis, oxidative burst) of the derived cells. It becomes apparent here that differentiation according to markers is misleading and could result in cells not able to perform expected functions after transplantation.

Co-culture with Living Brain Tissue: The differentiated microglia migrated, survived and showed function in living brain slices. However, they did not migrate deeply, but only in the immediate surface of the brain slices, although this could be caused by the surface injury induced by cutting the brain slices. This indicates that they could, in principle, if they invade the brain in circumstances that allow migration (Lesion, plaques, irradiation), perform the expected function. Differentiated microglia did, however, not ramify and down-regulate in co-culture. This could also be due to the injury induced by cutting the brain slices. Also, the harvesting (trypsinization) of cells could both hinder later ramification or activate the cells for a longer time.

Transplantation, Cell Tracking: First transplantations of microglia progenitors did result in detectable cells in the bone marrow, but not in the brain, showed up regulation of neurogenesis (Data not shown) but did not influence microglia activity in adult Alzheimer models significantly. A longer follow up period and transplantation in older animals might allow the detection of significant effects. Life long replenishment of microglia is hypothesized to take place at a slow rate. The detection of NA-BMCs in the bone marrow of non-irradiated, transplanted animals is encouraging - over a longer period of time cells could proliferate and slowly turn over microglia in the brain to a larger extent.

Stereology: We could measure changes in microglia activity and number with age using stereological methods. However, we could not detect a significant effect of our transplantations. Such stereological assessment of immune cell activity is time consuming but might offer an interesting additional method to cross check other measurements of inflammation. It allows to obtain absolute instead of relative results. Also, the differences between the age controls would quite certainly be more pronounced in older mice. Then, regulatory effects of transplanted cells could become more easily detectable.

8. Summary

Goals: Alzheimer and Multiple Sclerosis have been linked to a deterioration of the resident microglia cell population with age [1]. Therefore, we investigated here the differentiation of adult stem cells (mouse bone marrow) to microglia and potential application of these cells in a cell therapy.

Differentiation of Microglia: We screened several protocols for microglia differentiation. As the best protocol a culture of BM over 11 days, subsequent flushing off of non-adherent cells and differentiation in a combination of astrocyte-conditioned medium (ACM) and granulocyte/monocyte colony-stimulating factor (GM-CSF), emerged.

The cells showed long and branched processes and ramification, a morphology similar to primary microglia. They expressed antigens typical for primary microglia and macrophages. We could demonstrate that *in vitro* differentiated microglia are able to perform functions like phagocytosis and oxidative burst.

We noticed that our controls not supplemented with FMS-like tyrosine-kinase-3 ligand (Flt3L) were differentiating much better. The effect of Flt3L supplementation had not been the focus of previous papers [6]. This negative effect is however not surprising. The reported functions of Flt3L, namely hematopoietic stem cells (HSC) survival, adhesion and dendritic cell differentiation point to a possible interference with microglia differentiation.

Co-culture with Living Brain Tissue: We co-cultured the differentiated microglia with living brain slices. The cells survived and proliferated in co-culture. They also migrated into the immediate surface of the tissue. There are indications of phagocytic activity of the differentiated microglia in co-culture.

Transplantation, Cell Tracking and Stereology: We transplanted non-adherent bone marrow cells (‘Microglia progenitors’) and MSC intraveneously in young (2-3 months), adult (12 months) mice and Alzheimer models. We could detect NA-BMCs in bone marrow and lung but not in liver and brain.

The influence of cell transplantation on the activation of microglia in the brain was investigated using stereology. Stereological estimates show that NA-BMCs and MSCs do not regulate microglia activity significantly ($P = 0.08$).

We also wanted to investigate age related changes in general. We observed robust changes between young and adult control animals. The activity of microglia was significantly higher in adult animals compared to young animals. Such a higher MHC-II expression in aged microglia has also been observed by other groups using non-stereological methods [81].

The number of microglia increased significantly in adult female compared to young female mice. This has been reported previously in female, but not in male, mice. It was suggested that sex hormones or inflammation in age might cause this difference [24]. However, other groups reported a negative effect of these hormones on microglia proliferation [83]. Our results show that the density of microglia remained the same between adult and young animals and that the hippocampus volume increased. This indicates that normal growth of the brain takes place.

Outlook: We could show here that *in vitro* differentiated microglia are able to perform typical functions of primary microglia. They are phagocytic, perform oxidative burst, migrate into brain tissue and show morphology of primary microglia. *In vitro* derived microglia need to demonstrate the functional capacity of 'real' microglia cells and this research makes some contributions towards this aim. However, before such cells are deemed suitable and safe for transplantation extensive further tests will be required.

8. Zusammenfassung

Ziele: Alzheimer und Multiple Sklerose könnten mit einem Verfall und Funktionsverlust der residenten Mikroglia im Alter zusammenhängen [1]. Deshalb untersuchten wir hier die Differenzierung von adulten Stammzellen (Maus Knochenmark) zu Mikroglia und die potentielle Anwendung in einer Zelltherapie.

Differenzierung von Mikroglia: Wir verglichen experimentell eine Reihe von Protokollen zur Differenzierung von Mikroglia. Als bestes Protokoll stellte sich eine Kultur von Knochenmark über 11 Tage, nachfolgendes Abspülen nicht adherenter Zellen und deren Differenzierung in einer Kombination von Astrozyten-konditioniertem Medium (ACM) und Granulozyten/Monozyten Kolonien-stimulierenden Faktor (GM-CSF) heraus.

Die Zellen zeigten lange und verästelte Fortsätze, eine Morphologie ähnlich der von primären Mikroglia. Sie exprimierten typische Antigene primärer Mikroglia und

Makrophagen. Wir konnten zeigen dass *in vitro* differenzierte Mikroglia in der Lage zu Phagozytose und oxidativem Burst sind.

Wir bemerkten dass unsere Kontrollen, die nicht mit FMS-ähnliche Tyrosinkinase-3 Ligand (Flt3L) kultiviert wurden, viel besser differenzierten. Der Effekt von Flt3L Zugabe auf Mikroglia Differenzierung war nicht Fokus vorhergehender Arbeiten [6]. Dieser negative Effekt ist allerdings nicht überraschend. Die bekannten Funktionen von Flt3L, beispielsweise als Faktor für das Überleben von hämatopoietischen Stammzellen (HSC), Adhäsion und Differenzierung von dendritischen Zellen, weisen auf eine mögliche Beeinträchtigung der Differenzierng von Mikroglia hin.

Ko-Kultur mit Lebendem Hirngewebe: Wir ko-kultivierten die *in vitro* differenzierten Mikroglia zusammen mit lebendem Hirngewebe. Die Zellen überlebten und vermehrten sich in Ko-Kultur. Sie migrierten in die unmittelbare Oberfäche des Gewebes. Es gab auch Anzeichen für phagozytische Aktivität der *in vitro* differenzierten Mikroglia in Ko-Kultur.

Transplantation, Zelltracking und Stereologie: Wir transplantierten nicht-adherente Knochenmarkszellen (‘Mikroglia Vorläufer’) und MSC intravenös in junge (2-3 Monate), adulte (12 monate) und Alzheimer Mäuse. Wir konnten NA-BMCs in Knochenmark und Lunge aber nicht in Leber und Gehirn nachweisen.

Der Einfluss der Transplantationen auf die Aktivität von Mikroglia im Gehirn wurde mittels Stereologie gemessen. Stereologische Schätzungen zeigen dass NA-BMCs und MSCs die Aktivität von Mikroglia im Gehirn nicht signifikant beeinflussen ($P = 0.08$).

Wir wollten auch altersabhängige Veränderungen allgemein untersuchen. Wir bemerkten ausgeprägte Veränderungen zwischen jungen und adulten Kontrolltieren. Die Aktivität von Mikroglia im Gehirn war signifikant höher in adulten Tieren verglichen mit jungen. Solch eine höhere MHC-II Expression in gealterten Mikroglia wurde auch von anderen Gruppen mit nicht-stereologischen Methoden gemessen [81].

Die Zahl der Mikroglia im Hippocampus erhöhte sich signifikant in adulten weiblichen, verglichen mit jungen weiblichen, Mäusen. Dies wurde auch in der Vergangenheit in weiblichen, aber nicht in männlichen, Mäusen festgestellt. Dieser Unterschied könnte durch Sexualhormone oder Inflammation in fortgeschrittenem Alter verursacht werden [24]. Andere Gruppen berichteten allerdings dass solche Hormone einen negativen Effekt auf die Mikroglia Proliferation haben [83]. Unsere Ergebnisse zeigen

dass die Dichte von Mikroglia zwischen adulten und jungen Tieren gleich bleibt, allerdings das Hippocampus Volumen zunimmt. Das könnte ein Hinweis darauf sein dass normales Wachstum des Gehirns stattfindet.

Ausblick: Wir konnten hier zeigen dass *in vitro* differenzierte Mikroglia typische Funktionen von primären Mikroglia aufweisen. Sie sind phagozytisch, zeigen oxidativen Burst, Migration in lebendes Hirngewebe und Morphologie ähnlich der primärer Mikroglia. *In vitro* differenzierte Mikroglia müssen die Funktion 'echter' Microglia Zellen ausüben können und diese Arbeit leistet dazu einen Beitrag. Bevor die Zellen allerdings als geeignet und sicher für eine Transplantation angesehen werden können sind umfangreiche weitere Tests notwendig.

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10. Appendix

10.1. Abbreviations

ACM	Astrocyte Conditioned Medium
AFF488	Alexa Fluor 488
ALP	Alkaline Phosphatase
ASBI	6-Bromo-2-phospho-hydroxy-3-naphthoic acid o-anisidide
Balb/c	Mouse Strain
Balb/c SR/CR	Mouse Strain
BBB	Blood Brain Barrier
BDF1	Mouse Strain
BM	Bone Marrow
BMC	Bone Marrow Cells
BMT	Bone Marrow Transplantation
BV2	Microglia Cell Line
C57BL/6	Mouse Strain
CBA-J	Mouse Strain
CD	Cluster of Differentiation
CE	Coefficient of Error
CFU-f	Fibroblast Colony Forming Unit
CNS	Central Nervous System
CPS	Cryo Protection Solution
DAP12	DNAX Activating Protein 12kDa
DAPI	4',6-diamidino-2-phenylindole
DHR123	Dihydrorhodamin 123
DiO	3,3'-dioctadecyloxacarboxyanine perchlorate
DMEM	Dulbecco Minimal Essential Medium
DPBS	Dulbecco's Phosphate Buffered Saline
DPX	Distrene-80, Plasticizer, Xylene
ESC	Embryonic Stem Cells
FCS	Fetal Calf Serum
Flt3L	FMS-Like Tyrosine kinase 3 Ligand
FNA	Faciel Nerve Axotomy

FSC	Forward Scatter
FSN	Fluorescence Signal Noise Ratio
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte/Monocyte Colony Stimulating Factor
HBSS	Hank’s Balanced Salt Solution
HSC	Hematopoietic Stem Cells
HSPC	Hematopoietic Stem and Progenitor Cells
Iba-1	Ionized calcium-Binding Adapter molecule-1
IL	Interleukin
LPS	Lypopolysaccharides
M-CSF	Macrophage Colony Simulating Factor
MCP-1	Monocyte Chemoattractant Protein 1
MHC-II	Major Histocompatibility Complex II#
MP	Microglia Progenitors
MSC	Mesenchymal Stem Cells
NA-BMC	Non-Adherent Bone Marrow Cells
NADPH	Nicotinamidadenindinucleotid Phosphat
NBT	Nitro Blue Tetrazolium
NMDA	N-Methyl-D-Aspartic Acid
NO	Nitric Oxygen
NRBC	Nucleated Red Blood Cells
OHSC	Organotypic Hippocampal Slice Cultures
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFA	Paraformaldehyde
Pen/Strep	Penicillin/Streptomycin
PI	Propidium Iodide
PMA	Phorbol Myristic Acid
RPE	R-Phycoerythrin
ROS	Reactive Oxygen System
SCF	Stem Cell Factor
SE	Standard Error
SSC	Side Scatter
TGF-beta	Tumor Growth Factor beta

TLR	Toll Like Receptor
Tmem176b	Transmembrane Protein 176b
TNF-alpha	Tumor Necrosis Factor alpha
TREM2	Triggering Receptor
Tris	Tris (hydroxymethyl)-aminomethan

10.2. Independence Statement

1. I am aware of and have read the regulations concerning PhD theses of the Faculty for Biology and Pharmacy of the Friedrich Schiller University Jena
2. I created the here presented PhD thesis on my own. All sources, private communication and other help have been made plain.
3. All persons have been named who helped with the selection of the material and the creation of the manuscript.
4. The help of a PhD Advisor has not been enlisted.
5. Third persons have not directly or indirectly received money or equivalent benefits for work associated with the presented PhD thesis
6. The PhD thesis has not been handed in at another university. Also, it has not been handed in as thesis for a state examination or another scientific examination.

Arnd Hinze

25.02.2010, Leipzig

10.3. Author's Contributions

Arnd Hinze carried out all experiments and wrote the manuscripts. Alexandra Stolzing designed and coordinated the studies.

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